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<p>(54) Title: HUMAN CTLA-8 AND USES OF CTLA-8-RELATED PROTEINS</p> <p>(57) Abstract</p> <p>Polynucleotides encoding human CTLA-8 and related proteins are disclosed. Human CTLA-8 proteins and methods for their production are also disclosed. Methods of treatment using human CTLA-8 proteins, rat CTLA-8 proteins and herpesvirus herpes CTLA-8 proteins are also provided.</p>			

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HUMAN CTLA-8 AND USES OF CTLA-8-RELATED PROTEINS

This application is a continuation-in-part of application Ser. No. 08/504,032, filed July 19, 1995, and a continuation-in-part of application Ser. No. 08/514,014, filed August 11, 1995.

Field of the Invention

5 The present invention relates to human CTLA-8 proteins, nucleic acids encoding such proteins, methods of treatment using such proteins. The invention also relates to the use of rat CTLA-8 proteins and herpesvirus *Saimiri* ORF13 proteins in methods of treatment.

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Background of the Invention

Cytokines are secreted proteins which act on specific hematopoietic target cells to cause a differentiation event or on other target cells to induce a particular physiological response, such as secretion of proteins characteristic of inflammation. Cytokines, also variously known as lymphokines, hematopoietins, interleukins, colony stimulating factors, and the like, can be important therapeutic agents, especially for diseases or conditions in which a specific cell population is depleted. For example, erythropoietin, G-CSF, and GM-CSF, have all become important for treatment of anemia and leukopenia, respectively. Other cytokines such as interleukin-3, interleukin-6, interleukin-11 and interleukin-12 show promise in treatment of conditions such as thrombocytopenia and modulation of immune response.

For these reasons a significant research effort has been expended in searching for novel cytokines and cloning the DNAs which encode them. In the past, novel cytokines were identified by assaying a particular cell such as a bone marrow cell, for a measurable response, such as proliferation. The search for novel cytokines has thus been limited by the assays available, and if a novel cytokine has an activity which is unmeasurable by a known assay, the cytokine remains undetectable. In a newer approach, cDNAs encoding cytokines have been detected using the polymerase chain

reaction (PCR) and oligonucleotide primers having homology to shared motifs of known cytokines or their receptors. The PCR approach is also limited by the necessity for knowledge of previously cloned cytokines in the same protein family. Cytokines have also been cloned using subtractive hybridization to construct and screen cDNA libraries, or they can potentially be cloned using PCR followed by gel electrophoresis to detect differentially expressed genes. The subtractive hybridization methods are based on the assumption that cytokine mRNAs are those that are differentially expressed, and these methods do not require any prior knowledge of the sequence of interest. However, many cytokines may be encoded by mRNAs which are not differentially expressed, and thus are undetectable using these methods.

It would be desirable to develop new methods for identifying novel cytokines and other secreted factors and to isolate polynucleotides encoding them.

Summary of the Invention

In developing the present invention, methods were employed which selectively identify polynucleotides which encode secreted proteins. One such polynucleotide was isolated which encodes "human CTLA-8." In accordance with the present invention, polynucleotides encoding human CTLA-8 and active fragments thereof are disclosed. "CTLA-8" is used throughout the present specification to refer to both proteins and polynucleotides encoding those proteins and to refer to proteins and polynucleotides from all mammalian species.

In certain embodiments, the present invention provides an isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

- (a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 146 to nucleotide 544;
- (b) a nucleotide sequence capable of hybridizing to a nucleic acid sequence specified in (a);
- (c) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code; and
- (d) an allelic variant of the nucleotide sequence specified in (a).

Preferably, the polynucleotide of the invention encodes a protein having CTLA-8 activity. In other embodiments the polynucleotide is operably linked to an expression control sequence. In other preferred embodiments, the polynucleotide is contained in a vector suitable for *in vivo* expression in a mammalian subject. Polynucleotides comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 55 to nucleotide 544, the nucleotide sequence of SEQ ID NO:1 from nucleotide 139 to nucleotide 544 or the nucleotide sequence of SEQ ID NO:1 from nucleotide 86 to nucleotide 544 are particularly preferred.

Host cells transformed with the polynucleotides of the invention are also provided, including mammalian cells.

Processes are also provided for producing a human CTLA-8 protein, said processes comprising:

- (a) growing a culture of the host cell of the invention in a suitable culture medium; and
- 15 (b) purifying the human CTLA-8 protein from the culture.

Isolated human CTLA-8 protein is also provided which comprising an amino acid sequence selected from the group consisting of:

- (a) the amino acid sequence of SEQ ID NO:2;
- (b) the amino acid sequence of SEQ ID NO:2 from amino acids 11 to 163;
- 20 (c) the amino acid sequence of SEQ ID NO:2 from amino acids 29 to 163;
- (d) the amino acid sequence of SEQ ID NO:2 from amino acids 31 to 163;
and
- (e) fragments of (a), (b), (c) or (d) having CTLA-8 activity.

25 Proteins comprising the amino acid sequence of SEQ ID NO:2 and comprising the sequence from amino acids 29 to 163, from amino acid 31 to 163, or from amino acids 11 to 163 of SEQ ID NO:2 are particularly preferred. Preferably, the protein has CTLA-8 activity. Pharmaceuticals composition comprising a human CTLA-8 protein of the invention and a pharmaceutically acceptable carrier are also provided.

30 Compositions are also disclosed which comprise an antibody which specifically reacts with a human CTLA-8 protein of the invention.

Methods of treating a mammalian subject are also provided which comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a human CTLA-8 protein.

5 Rat CTLA-8 and active (*i.e.*, having CTLA-8 activity) fragments thereof may also be used in such methods of treatment. Preferably the rat protein is administered as a composition comprising a pharmaceutically acceptable carrier and a protein comprising an amino acid sequence selected from the group consisting of:

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- (a) the amino acid sequence of SEQ ID NO:4;
- (b) the amino acid sequence of SEQ ID NO:4 from amino acids 18 to 150; and
- (c) fragments of (a) or (b) having CTLA-8 activity.

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Herpesvirus *Saimiri* ORF13, referred to herein as "herpes CTLA-8", and active (*i.e.*, having CTLA-8 activity) fragments thereof and active fragments thereof may also be used in such methods of treatment. Preferably the herpes CTLA-8 protein is administered as a composition comprising a pharmaceutically acceptable carrier and a protein comprising an amino acid sequence selected from the group consisting of:

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- (a) the amino acid sequence of SEQ ID NO:6;
- (b) the amino acid sequence of SEQ ID NO:6 from amino acids 19 to 151; and
- (c) fragments of (a) or (b) having CTLA-8 activity.

The invention also provides a method of treating a mammalian subject comprising administering a therapeutically effective amount of a composition comprising a pharmaceutically acceptable carrier and IL-17 or an active fragment thereof.

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In methods of treatment provided by the present invention, preferably the subject is treated to produce an effect selected from the group consisting of inhibition of angiogenesis, inhibition of growth or proliferation of vascular endothelial cells, inhibition of tumor growth, inhibition of angiogenesis-dependent tissue growth, proliferation of myeloid cells or progenitors, proliferation of erythroid cells or progenitors, proliferation of lymphoid cells or progenitors, induction of IFN γ production, induction of IL-3 production and induction of GM-CSF production.

Brief Description of the Figures

Fig. 1 is a comparison of homologous regions of the amino acid sequences of human CTLA-8 (indicated as "B18_F1"), rat CTLA-8 (indicated as "Musctla8") and herpes CTLA-8 (indicated as "Hsvie_2").

5 Fig. 2 depicts autoradiographs demonstrating expression of human CTLA-8 in COS cells.

Fig. 3 presents data relating to the ability of human CTLA-8 to inhibit angiogenesis.

10 Figs. 4 and 5 present data relating to the ability of human CTLA-8 to produce or induce hematopoietic activity.

Figs. 6 and 7 present data demonstrating the ability of human CTLA-8 to induce production of IL-6 and IL-8.

Detailed Description of Preferred Embodiments

15 The inventors of the present application have identified and provided a polynucleotide encoding a human CTLA-8 protein. SEQ ID NO:1 provides the nucleotide sequence of a cDNA encoding the human CTLA-8 protein. SEQ ID NO:2 provides the amino acid sequence of the human CTLA-8 protein. Alternatively, the initiating methionine may be at amino acid 11 of SEQ ID NO:2. On the basis of 20 amino terminal sequencing, the mature protein sequence is believed to begin at amino acid 31 of SEQ ID NO:2 (encoded by the sequence beginning with nucleotide 146 of SEQ ID NO:1).

The region from amino acid 29 to amino acid 163 of human CTLA-8 (SEQ ID NO:2) shows marked homology to portions of rat CTLA-8 (amino acids 18 to 150 of 25 SEQ ID NO:4) and herpesvirus *Saimiri* ORF13 ("herpes CTLA-8") (amino acids 19 to 151 of SEQ ID NO:5). A cDNA sequence encoding rat CTLA-8 is listed at SEQ ID NO:3 and its corresponding amino acid sequence is reported at SEQ ID NO:4. A cDNA sequence encoding herpes CTLA-8 is listed at SEQ ID NO:5 and its corresponding amino acid sequence is reported at SEQ ID NO:6. Homology between 30 rat CTLA-8 and herpes CTLA-8 was reported by Rouvier et al., J. Immunol. 1993, 150, 5445-5456.

Applicants had previously incorrectly identified the rat sequences of SEQ ID NO:3 and SEQ ID NO:4 as applying to murine CTLA-8. Applicants' human CTLA-8 (B18) does also show homology to the true murine CTLA-8 sequence.

5 Golstein et al. (WO95/18826; Fossiez et al., Microbial Evasion and Subversion of Immunity 544:3222 (Abstract)) have also reported a species they initially identified as "human CTLA-8." However, examination of the sequence of the Golstein et al. species and the human CTLA-8 (B18) sequence of the present invention readily reveals that they are two different proteins, although they are homologous with each other and with the rat CTLA-8 and herpes CTLA-8 identified herein. The Golstein 10 et al. species has now been renamed as interleukin-17 (IL-17). Because of the homology between applicants' human CTLA-8 (B18) and IL-17, these proteins are expected to share some activities.

15 It has also been preliminarily determined that human CTLA-8 (B18) forms homodimers when expressed. As a result, human CTLA-8 proteins may possess activity in either monomeric or dimeric forms. Human CTLA-8 proteins can also be produced as heterodimers with rat and herpes CTLA-8 proteins and with human IL-17. These heterodimers are also expected to have activities of the proteins of which they are comprised.

20 Forms of human CTLA-8 protein of less than full length are encompassed within the present invention and may be produced by expressing a corresponding fragment of the polynucleotide encoding the human CTLA-8 protein (SEQ ID NO:1). These corresponding polynucleotide fragments are also part of the present invention. Modified polynucleotides as described above may be made by standard molecular biology techniques, including site-directed mutagenesis methods which are known in 25 the art or by the polymerase chain reaction using appropriate oligonucleotide primers.

For the purposes of the present invention, a protein has "CTLA-8 activity" if it either (1) displays biological activity in a factor-dependent cell proliferation assay (preferably an assay in which full-length the corresponding species full-length CTLA-8 is active) (including without limitation those assays described below), or (2) induces expression or secretion of γ -IFN, or (3) displays chemoattractant or chemotactic 30 activity in a chemoattraction or chemotaxis assay (preferably as assay in which full-

length the corresponding species full-length CTLA-8 is active) or (4) induces expression of secretion of IL-3 or GM-CSF.

5 Human CTLA-8 protein or fragments thereof having CTLA-8 activity may be fused to carrier molecules such as immunoglobulins. For example, human CTLA-8 protein may be fused through "linker" sequences to the Fc portion of an immunoglobulin.

The invention also encompasses allelic variations of the nucleotide sequence as set forth in SEQ ID NO:1, that is, naturally-occurring alternative forms of the isolated polynucleotide of SEQ ID NO:1 which also encode human CTLA-8 or 10 CTLA-8 proteins having CTLA-8 activity. Also included in the invention are isolated polynucleotides which hybridize to the nucleotide sequence set forth in SEQ ID NO:1 under highly stringent (0.2xSSC at 65°C), stringent (e.g. 4xSSC at 65 C or 50% formamide and 4xSSC at 42°C), or relaxed (4xSSC at 50°C or 30-40% formamide and 4xSSC at 42°C) conditions. Isolated polynucleotides which encode human CTLA-8 15 protein but which differ from the nucleotide sequence set forth in SEQ ID NO:1 by virtue of the degeneracy of the genetic code are also encompassed by the present invention. Variations in the nucleotide sequence as set forth in SEQ ID NO:1 which are caused by point mutations or by induced modifications which enhance CTLA-8 activity, half-life or production level are also included in the invention.

20 The isolated polynucleotides of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the CTLA-8 protein recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant proteins are also known 25 and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means enzymatically or chemically ligated to form a covalent bond between the isolated polynucleotide of the invention and the expression control sequence, in such a way that the CTLA-8 protein is expressed by a host cell which has been transformed (transfected) with the ligated 30 polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the human CTLA-8 protein. Any cell type capable of expressing functional human CTLA-8 protein may be used. Suitable mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, 5 human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK, Rat2, BaF3, 32D, FDCP-1, PC12 or C2C12 cells.

The human CTLA-8 protein may also be produced by operably linking the 10 isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, California, U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas 15 Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference. Soluble forms of the human CTLA-8 protein may also be produced in insect cells using appropriate isolated polynucleotides as described above.

Alternatively, the human CTLA-8 protein may be produced in lower 20 eukaryotes such as yeast or in prokaryotes such as bacteria. Suitable yeast strains include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, *Candida*, or any yeast strain capable of expressing heterologous proteins. Suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing heterologous proteins.

The human CTLA-8 protein of the invention may also be expressed as a 25 product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide sequence encoding the human CTLA-8 protein.

The human CTLA-8 protein of the invention may be prepared by growing a 30 culture of transformed host cells under culture conditions necessary to express the desired protein. The resulting expressed protein may then be purified from the culture medium or cell extracts. Soluble forms of the human CTLA-8 protein of the invention

can be purified from conditioned media. Membrane-bound forms of human CTLA-8 protein of the invention can be purified by preparing a total membrane fraction from the expressing cell and extracting the membranes with a non-ionic detergent such as Triton X-100.

5 The human CTLA-8 protein can be purified using methods known to those skilled in the art. For example, the human CTLA-8 protein of the invention can be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium.

10 Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the human CTLA-8 protein from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether,

15 or propyl ether; or by immunoaffinity chromatography. Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the human CTLA-8 protein. Some or all of the foregoing purification steps, in various combinations or with other known methods, can also be employed to provide a substantially purified isolated recombinant protein.

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Preferably, the human CTLA-8 protein is purified so that it is substantially free of other mammalian proteins.

It is believed that human CTLA-8, active fragments and variants thereof, and
30 CTLA-8 related proteins (such as, for example, rat CTLA-8 and herpes CTLA-8) (collectively "CTLA-8 proteins") possess or induce cytokine activities. Human

5 CTLA-8 expression correlated with γ -IFN expression in induced primary cells and can induce the expression of IL-3 and/or GM-CSF, which expression can in turn produce effects associated with the induced cytokine. Therefore, human CTLA-8 and CTLA-8 related proteins may have an effect on proliferation or function of myeloid cells, erythroid cells, lymphoid cells and their progenitors. Human CTLA-8 proteins may also play a role in formation of platelets or their progenitors.

10 A protein of the present invention may exhibit cytokine, cell proliferation (either inducing or inhibiting) or cell differentiation (either inducing or inhibiting) activity or may induce production of other cytokines in certain cell populations. Many protein factors discovered to date, including all known cytokines, have exhibited activity in one or more factor dependent cell proliferation assays, and hence the assays serve as a convenient confirmation of cytokine activity. The activity of a protein of the present invention is evidenced by any one of a number of routine factor dependent cell proliferation assays for cell lines including, without limitation, 32D, DA2, DA1G, 15 T10, B9, B9/11, BaF3, MC9/G, M+ (preB M+), 2E8, RB5, DA1, 123, T1165, HT2, CTLL2, TF-1, Mo7e and CMK.

The activity of a protein of the invention may, among other means, be measured by the following methods:

20 Assays for T-cell or thymocyte proliferation include without limitation those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Takai et al., J. Immunol. 137:3494-3500, 1986; Bertagnolli et al., J. Immunol. 145:1706-1712, 1990; 25 Bertagnolli et al., Cellular Immunology 133:327-341, 1991; Bertagnolli, et al., J. Immunol. 149:3778-3783, 1992; Bowman et al., J. Immunol. 152: 1756-1761, 1994.

30 Assays for cytokine production and/or proliferation of spleen cells, lymph node cells or thymocytes include, without limitation, those described in: Polyclonal T cell stimulation, Kruisbeek, A.M. and Shevach, E.M. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 3.12.1-3.12.14, John Wiley and Sons, Toronto. 1994; and Measurement of mouse and human Interferon γ , Schreiber, R.D.

In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.8.1-6.8.8, John Wiley and Sons, Toronto. 1994.

Assays for proliferation and differentiation of hematopoietic and lymphopoietic cells include, without limitation, those described in: Measurement of Human and Murine Interleukin 2 and Interleukin 4, Bottomly, K., Davis, L.S. and Lipsky, P.E. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.3.1-6.3.12, John Wiley and Sons, Toronto. 1991; deVries et al., J. Exp. Med. 173:1205-1211, 1991; Moreau et al., Nature 336:690-692, 1988; Greenberger et al., Proc. Natl. Acad. Sci. U.S.A. 80:2931-2938, 1983; Measurement of mouse and human interleukin 6 - Nordan, R. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.6.1-6.6.5, John Wiley and Sons, Toronto. 1991; Smith et al., Proc. Natl. Acad. Sci. U.S.A. 83:1857-1861, 1986; Measurement of human Interleukin 11 - Bennett, F., Giannotti, J., Clark, S.C. and Turner, K. J. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.15.1 John Wiley and Sons, Toronto. 1991; Measurement of mouse and human Interleukin 9 - Ciarletta, A., Giannotti, J., Clark, S.C. and Turner, K.J. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.13.1, John Wiley and Sons, Toronto. 1991.

Assays for T-cell clone responses to antigens (which will identify, among others, proteins that affect APC-T cell interactions as well as direct T-cell effects by measuring proliferation and cytokine production) include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function; Chapter 6, Cytokines and their cellular receptors; Chapter 7, Immunologic studies in Humans); Weinberger et al., Proc. Natl. Acad. Sci. USA 77:6091-6095, 1980; Weinberger et al., Eur. J. Immun. 11:405-411, 1981; Takai et al., J. Immunol. 137:3494-3500, 1986; Takai et al., J. Immunol. 140:508-512, 1988.

A protein of the present invention may also exhibit immune stimulating or immune suppressing activity, including without limitation the activities for which assays are described herein. A protein may be useful in the treatment of various

immune deficiencies and disorders (including severe combined immunodeficiency (SCID)), e.g., in regulating (up or down) growth and proliferation of T and/or B lymphocytes, as well as effecting the cytolytic activity of NK cells and other cell populations. These immune deficiencies may be genetic or be caused by viral (e.g.,
5 HIV) as well as bacterial or fungal infections, or may result from autoimmune disorders. More specifically, infectious diseases causes by viral, bacterial, fungal or other infection may be treatable using a protein of the present invention, including infections by HIV, hepatitis viruses, herpes viruses, mycobacteria, leshmania, malaria and various fungal infections such as candida. Of course, in this regard, a protein of
10 the present invention may also be useful where a boost to the immune system generally would be indicated, i.e., in the treatment of cancer.

Autoimmune disorders which may be treated using a protein of the present invention include, for example, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, autoimmune pulmonary inflammation, Guillain-Barre syndrome,
15 autoimmune thyroiditis, insulin dependent diabetes mellitis, myasthenia gravis, graft-versus-host disease and autoimmune inflammatory eye disease. Such a protein of the present invention may also be useful in the treatment of allergic reactions and conditions, such as asthma or other respiratory problems. Other conditions, in which immune suppression is desired (including, for example, asthma and related respiratory
20 conditions), may also be treatable using a protein of the present invention.

A protein of the present invention may also suppress chronic or acute inflammation, such as, for example, that associated with infection (such as septic shock or systemic inflammatory response syndrome (SIRS)), inflammatory bowel disease, Crohn's disease or resulting from over production of cytokines such as TNF
25 or IL-1 (such as the effect demonstrated by IL-11).

The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for thymocyte or splenocyte cytotoxicity include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan,
30 A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte

Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Herrmann et al., Proc. Natl. Acad. Sci. USA 78:2488-2492, 1981; Herrmann et al., J. Immunol. 128:1968-1974, 1982; Handa et al., J. Immunol. 135:1564-1572, 1985; Takai et al., J. Immunol. 137:3494-3500, 1986; Takai et al., J. Immunol. 140:508-512, 1988;

5 Herrmann et al., Proc. Natl. Acad. Sci. USA 78:2488-2492, 1981; Herrmann et al., J. Immunol. 128:1968-1974, 1982; Handa et al., J. Immunol. 135:1564-1572, 1985; Takai et al., J. Immunol. 137:3494-3500, 1986; Bowman et al., J. Virology 61:1992-1998; Takai et al., J. Immunol. 140:508-512, 1988; Bertagnolli et al., Cellular Immunology 133:327-341, 1991; Brown et al., J. Immunol. 153:3079-3092, 1994.

10 Assays for T-cell-dependent immunoglobulin responses and isotype switching (which will identify, among others, proteins that modulate T-cell dependent antibody responses and that affect Th1/Th2 profiles) include, without limitation, those described in: Maliszewski, J. Immunol. 144:3028-3033, 1990; and Assays for B cell function: *In vitro* antibody production, Mond, J.J. and Brunswick, M. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 3.8.1-3.8.16, John Wiley and Sons, Toronto. 1994.

15 Mixed lymphocyte reaction (MLR) assays (which will identify, among others, proteins that generate predominantly Th1 and CTL responses) include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Takai et al., J. Immunol. 137:3494-3500, 1986; Takai et al., J. Immunol. 140:508-512, 1988; Bertagnolli et al., J. Immunol. 149:3778-3783, 1992.

20 Dendritic cell-dependent assays (which will identify, among others, proteins expressed by dendritic cells that activate naive T-cells) include, without limitation, those described in: Guery et al., J. Immunol. 134:536-544, 1995; Inaba et al., Journal of Experimental Medicine 173:549-559, 1991; Macatonia et al., Journal of Immunology 154:5071-5079, 1995; Porgador et al., Journal of Experimental Medicine 182:255-260, 1995; Nair et al., Journal of Virology 67:4062-4069, 1993; Huang et al., Science 264:961-965, 1994; Macatonia et al., Journal of Experimental Medicine

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169:1255-1264, 1989; Bhardwaj et al., Journal of Clinical Investigation 94:797-807, 1994; and Inaba et al., Journal of Experimental Medicine 172:631-640, 1990.

Assays for lymphocyte survival/apoptosis (which will identify, among others, proteins that prevent apoptosis after superantigen induction and proteins that regulate lymphocyte homeostasis) include, without limitation, those described in: Darzynkiewicz et al., Cytometry 13:795-808, 1992; Gorczyca et al., Leukemia 7:659-670, 1993; Gorczyca et al., Cancer Research 53:1945-1951, 1993; Itoh et al., Cell 66:233-243, 1991; Zacharchuk, Journal of Immunology 145:4037-4045, 1990; Zamai et al., Cytometry 14:891-897, 1993; Gorczyca et al., International Journal of Oncology 1:639-648, 1992.

Assays for proteins that influence early steps of T-cell commitment and development include, without limitation, those described in: Antica et al., Blood 84:111-117, 1994; Fine et al., Cellular Immunology 155:111-122, 1994; Galy et al., Blood 85:2770-2778, 1995; Toki et al., Proc. Nat. Acad. Sci. USA 88:7548-7551, 1991.

A protein of the present invention may be useful in regulation of hematopoiesis and, consequently, in the treatment of myeloid or lymphoid cell deficiencies. Even marginal biological activity in support of colony forming cells or of factor-dependent cell lines indicates involvement in regulating hematopoiesis, e.g. in supporting the growth and proliferation of erythroid progenitor cells alone or in combination with other cytokines, thereby indicating utility, for example, in treating various anemias or for use in conjunction with irradiation/chemotherapy to stimulate the production of erythroid precursors and/or erythroid cells; in supporting the growth and proliferation of myeloid cells such as granulocytes and monocytes/macrophages (i.e., traditional CSF activity) useful, for example, in conjunction with chemotherapy to prevent or treat consequent myelo-suppression; in supporting the growth and proliferation of megakaryocytes and consequently of platelets thereby allowing prevention or treatment of various platelet disorders such as thrombocytopenia, and generally for use in place of or complimentarily to platelet transfusions; and/or in supporting the growth and proliferation of hematopoietic stem cells which are capable of maturing to any and all of the above-mentioned hematopoietic cells and therefore find therapeutic utility

in various stem cell disorders (such as those usually treated with transplantation, including, without limitation, aplastic anemia and paroxysmal nocturnal hemoglobinuria), as well as in repopulating the stem cell compartment post irradiation/chemotherapy, either *in-vivo* or *ex-vivo* (i.e. in conjunction with bone marrow transplantation) as normal cells or genetically manipulated for gene therapy.

5 The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for proliferation and differentiation of various hematopoietic lines are cited above.

10 Assays for embryonic stem cell differentiation (which will identify, among others, proteins that influence embryonic differentiation hematopoiesis) include, without limitation, those described in: Johansson et al. *Cellular Biology* 15:141-151, 1995; Keller et al., *Molecular and Cellular Biology* 13:473-486, 1993; McClanahan et al., *Blood* 81:2903-2915, 1993.

15 Assays for stem cell survival and differentiation (which will identify, among others, proteins that regulate lympho-hematopoiesis) include, without limitation, those described in: Methylcellulose colony forming assays, Freshney, M.G. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 265-268, Wiley-Liss, Inc., New York, NY. 1994; Hirayama et al., *Proc. Natl. Acad. Sci. USA* 89:5907-5911, 1992; Primitive hematopoietic colony forming cells with high proliferative potential, McNiece, I.K. and Briddell, R.A. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 23-39, Wiley-Liss, Inc., New York, NY. 1994; Neben et al., *Experimental Hematology* 22:353-359, 1994; Cobblestone area forming cell assay, Ploemacher, R.E. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 1-21, Wiley-Liss, Inc., New York, NY. 1994; Long term bone marrow cultures in the presence of stromal cells, Spooncer, E., Dexter, M. and Allen, T. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 163-179, Wiley-Liss, Inc., New York, NY. 1994; Long term culture initiating cell assay, Sutherland, H.J. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 139-162, Wiley-Liss, Inc., New York, NY. 1994.

CTLA-8 proteins are useful in the treatment of various immune deficiencies and disorders (including SCID), *e.g.*, in regulating (up or down) growth, proliferation and/or activity of T and/or B lymphocytes, as well as the cytolytic activity of NK cells. These immune deficiencies may be caused by viral (*e.g.*, HIV) as well as bacterial infections, or may result from autoimmune disorders. More specifically, infectious diseases caused by viral, bacterial, fungal or other infection may be treatable using CTLA-8 proteins, including infections by HIV, hepatitis, influenza, CMV, herpes, mycobacterium, leishmaniasis, malaria and various fungal infections (such as candida). Of course, in this regard, the CTLA-8 proteins may also be useful where a boost to the immune system generally would be indicated, *i.e.*, in the treatment of cancer or as an adjuvant to vaccines. Autoimmune disorders which may be treated using factors of the present invention include, for example, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, autoimmune pulmonary inflammation, Guillain-Barre syndrome, autoimmune thyroiditis, insulin dependent diabetes melitis and autoimmune inflammatory eye disease. The CTLA-8 proteins are also expected to be useful in the treatment of allergic reactions and conditions.

CTLA-8 proteins are also expected to have chemotactic activity. A protein or peptide has "chemotactic activity," as used herein, if it can stimulate, directly or indirectly, the directed orientation or movement of cells, including myeloid and lymphoid cells. Preferably, the protein or peptide has the ability to directly stimulate directed movement of cells (particularly T-cells). Whether a particular protein or peptide has chemotactic activity for cells can be readily determined by employing such protein or peptide in any known assay for cell chemotaxis.

CTLA-8 proteins also inhibit growth and proliferation of vascular endothelial cells. As a result, human CTLA-8 proteins are effective in inhibiting angiogenesis (*i.e.*, vascular formation). This activity will also be useful in the treatment of tumors and other conditions in which angiogenesis is involved. Inhibition of angiogenesis by human CTLA-8 proteins will also result in inhibition or prevention of the condition to which normal angiogenesis would contribute.

Isolated CTLA-8 proteins, purified from cells or recombinantly produced, may be used as a pharmaceutical composition when combined with a pharmaceutically

acceptable carrier. Such a composition may contain, in addition to CTLA-8 protein and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, G-CSF, γ -IFN, stem cell factor, and erythropoietin. The pharmaceutical composition may contain thrombolytic or anti-thrombotic factors such as plasminogen activator and Factor VIII. The pharmaceutical composition may further contain other anti-inflammatory agents. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with CTLA-8 protein, or to minimize side effects caused by the CTLA-8 protein. Conversely, CTLA-8 protein may be included in formulations of the particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which CTLA-8 protein is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, e.g., amelioration of symptoms of,

healing of, or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in 5 combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of CTLA-8 protein is administered to a mammal. CTLA-8 protein may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing 10 cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines, other hematopoietic factors or vaccine components (such as antigens or other adjuvants), CTLA-8 protein may be administered either simultaneously with the cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If 15 administered sequentially, the attending physician will decide on the appropriate sequence of administering CTLA-8 protein in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

Administration of CTLA-8 protein used in the pharmaceutical composition or 20 to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of CTLA-8 protein is administered orally, CTLA-8 protein will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the 25 invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% CTLA-8 protein, and preferably from about 25 to 90% CTLA-8 protein. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid 30 form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol,

propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of CTLA-8 protein, and preferably from about 1 to 50% CTLA-8 protein.

When a therapeutically effective amount of CTLA-8 protein is administered
5 by intravenous, cutaneous or subcutaneous injection, CTLA-8 protein will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in
10 addition to CTLA-8 protein an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

15 The amount of CTLA-8 protein in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of CTLA-8 protein with which to treat each individual patient. Initially, the attending physician will
20 administer low doses of CTLA-8 protein and observe the patient's response. Larger doses of CTLA-8 protein may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not generally increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.1 µg to about 100 mg of
25 CTLA-8 protein per kg body weight, preferably about 0.1 µg to about 10 mg of CTLA-8 protein per kg body weight, more preferably about 0.1 µg to about 100 µg of CTLA-8 protein per kg body weight, most preferably preferably about 0.1 µg to about 10 µg of CTLA-8 protein per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of
30 the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It

is contemplated that the duration of each application of the CTLA-8 protein will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

5 CTLA-8 protein of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the CTLA-8 protein and which may inhibit CTLA-8 binding to its receptor. Such antibodies are also useful for performing diagnostics assays for CTLA-8 in accordance with known methods. Such antibodies may be obtained using the entire CTLA-8 protein as an 10 immunogen, or by using fragments of human CTLA-8 protein. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Additional peptide immunogens may be generated by replacing tyrosine residues with sulfated tyrosine residues. Methods for synthesizing such peptides are known in the art, for 15 example, as in R.P. Merrifield, J.Amer.Chem.Soc. 85, 2149-2154 (1963); J.L. Krstenansky, et al., FEBS Lett. 211, 10 (1987).

Neutralizing or non-neutralizing antibodies (preferably monoclonal antibodies) binding to human CTLA-8 protein may also be useful therapeutics for certain tumors and also in the treatment of conditions described above. These neutralizing 20 monoclonal antibodies are capable of blocking the ligand binding to the human CTLA-8 protein or may promote clearance of protein from the patient.

Because of their homology to human CTLA-8, rat CTLA-8 proteins, herpes 25 CTLA-8 proteins and IL-17 proteins (the "human CTLA-8" of Golstein et al., *supra*) will also possess CTLA-8 activity as described above. As a result, rat and herpes CTLA-8 proteins and IL-17 proteins, as well as active fragments and variants thereof, can be used in preparation of pharmaceutical compositions and in methods of treatment as described for human CTLA-8. Rat and herpes CTLA-8 proteins, and active fragments and variants thereof, can be produced as described above using the 30 polynucleotides (or fragments or variants thereof) described in SEQ ID NO:3 and SEQ ID NO:5, respectively. Rat and herpes CTLA-8 may also be produced as described in Rouvier et al., *J. Immunol.* 1993, 150, 5445-5456. CTLA-8 proteins of other

species can also be used as described herein. cDNAs encoding rat CTLA-8 and herpes CTLA-8 were deposited with the American Type Culture Collection on July 6, 1995 and assigned accession numbers ATCC 69867 and ATCC 69866, respectively. IL-17 proteins may also be produced as described in Golstein et al., *supra*.

5 Because of its homology to IL-17, the human CTLA-8 (B18) proteins of the present invention may also share some activities with IL-17.

For the purposes of treatment or therapy, any of the proteins discussed or disclosed herein may be administered by *in vivo* expression of the protein in a mammalian subject. In such instances, a polynucleotide encoding the desired protein 10 is administered to the subject in manner allowing expression in accordance with known methods, including without limitation the adenovirus methods disclosed herein.

Example 1

Isolation of Human CTLA-8 cDNA

A partial clone for human CTLA-8 was isolated from a cDNA library made from RNA isolated from stimulated human peripheral blood mononuclear cells. This partial was identified as "B18." B18 is sometimes used herein to refer to the human CTLA-8 of the present invention. Homology searches identified this partial clone as 20 being related to the herpes and rat CTLA-8 genes. DNA sequence of this partial clone was used to isolate the full-length clone.

In order to isolate a full-length cDNA for B18, a directional, full-length cDNA library by standard means in the COS expression vector pMV2. The cDNA library was transformed into *E. coli* by electroporation. The bulk of the original transformed 25 cDNA library was frozen in glycerol at -80°C. An aliquot was titered to measure the concentration of transformed *E. coli*. The *E. coli* were thawed, diluted to 76,000/0.1 ml in media containing ampicillin, and 0.1 ml was distributed into the wells of a microtiter dish in an 8 x 8 array. The microtiter dish was placed at 37°C overnight to grow the *E. coli*.

30 To prepare DNA for PCR, 20 µl aliquots of culture from each well were withdrawn and pooled separately for each row and column of eight wells, giving 16

pools of 160 µl each. The *E. coli* were pelleted, resuspended in 160 µl of standard lysis buffer consisting of 10 mM TrisHCl pH8, 1 mM EDTA, 0.01% Triton X-100, and lysed by heating to 95°C for 10 minutes.

To identify which of the wells contained *E. coli* transformed with B18, PCR
5 was performed first on the DNA preps corresponding to the eight columns. The PCR consisted of two sequential reactions with nested oligonucleotides using standard conditions. The oligonucleotides used for the PCR reaction were derived from the sequence of the partial B18 clone. They were:

10 B185: CACAGGCATACACAGGAAGATACTTCA (SEQ ID NO:7)
 B183: TCTTGCTGGATGGGAACGGAATTCA (SEQ ID NO:8)
 B18N: ATACATTCACAGAAGAGCTTCCTGCACA (SEQ ID NO:9)

The PCR conditions were 2.5 mM MgCl₂ and 95°C x 2 min for one cycle, 95°C x 1
15 min plus 68°C x 1 min for 30 cycles, and 68°C x 10 for one cycle. Each reaction was 20 µl. The first reaction contained oligonucleotides B185 and B183 and 1µl of the DNA preparations. The second reaction contained oligonucleotides B183 and B18N and 1 µl of the first reaction.

DNA preps that potentially contained a full-length B18 cDNA clone were
20 identified by agarose gel electrophoresis on an aliquot of the second PCR reaction. A DNA band of the correct mobility was assumed to be derived from a B18 cDNA. Next the same sequence of PCR reactions and gel analysis was done on the DNA preps corresponding to the eight rows. The intersection of a row and a column identified well A2 as potentially containing B18, narrowing it down to the 76,000 *E.*
25 *coli* originally seeded into that well.

To further purify the individual *E. coli* containing the putative full-length B18 cDNA clone, the concentration of *E. coli* in well A2 was measured by titering and plating dilutions of the well. Then 7600 *E. coli* were seeded into the wells of a second microtiter plate in an 8 x 8 array. The *E. coli* were grown overnight; wells were
30 pooled, and DNA was prepared as described above. To identify which of these wells contained *E. coli* transformed with B18, sequential PCR reactions were performed

essentially as described above. Agarose gel electrophoresis identified well B2 as potentially containing a B18 cDNA.

The *E. coli* containing this cDNA was further purified by seeding wells of a microtiter plate with 253 *E. coli* per well and proceeding as for the purification of the 5 *E. coli* in well A2. Well C3 was identified as containing a putative full-length B18 cDNA clone. The exact *E. coli* was identified by plating the contents of the well onto bacterial culture media and then screening the *E. coli* colonies following established protocols. The probe for these hybridizations was a PCR fragment generated by doing a PCR reaction on the B18 clone using as primers the oligonucleotides described 10 above (SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9). When a single colony was identified, DNA was prepared and sequenced by standard methods. Comparison of this sequence to the sequence of the original partial clone confirmed identity and that the isolated cDNA was full-length.

The full-length clone was deposited with the American Type Culture 15 Collection on July 6, 1995 and assigned accession number ATCC 69868.

Example 2

Expression of Human CTLA-8

The full-length B18 clone for human CTLA-8 was transfected into COS cells 20 which were then labelled with ^{35}S -methionine. An aliquot of conditioned medium from the transfected cell culture was reduced, denatured and electrophoresed on polyacrylamide gels. Autoradiographs of those gels are reproduced in Fig. 2. The band indicated by the arrow demonstrates expression of human CTLA-8.

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Example 3

Inhibition of Angiogenesis by Human CTLA-8

The ability of human CTLA-8 to inhibit angiogenesis was examined in an angiostatic activity assay (endothelial cell proliferation assay). The assay was done in a 96 well plate. Primary human umbilical cells (HUVECs) were seeded to 2×10^3 30 cells per well in EGM medium (Clonetics)/20% FCS and incubated at 37°C for 24 hr. The cells were then starved in M199 medium (GIBCO BRL) containing 10% charcoal

treated serum (M199-CS) for 48 hr at 37°C. Conditioned media containing B18 (human CTLA-8) was obtained from transfected COS or stably expressing CHO cells and 1:10, 1:50, 1:250, and 1:1250 dilutions prepared in M199-CS medium containing 100 ng/ml FGF. The dilutions of B18 were added to the starved cells and incubated 5 for 72 hr at 37°C. The cells were then radiolabeled by [³H]-thymidine for 6 hr. Radiolabeled cells were washed with PBS and trypsinized for liquid scintillation counting. Results were plotted using Kaleidograph software. The results are shown in Fig. 3. In the figure, "Med" is the mock control, "B18" and "B18-1" were conditioned medium from two independent transfections of COS with DNA encoding 10 human CTLA-8 (B18). IFN γ was used as a positive control angiostatic (i.e., angiogenesis inhibition) activity. These data demonstrate that human CTLA-8 (B18) inhibits angiogenesis.

Example 4

15

Hematopoietic Activity of Human CTLA-8

The hematopoietic activity of human CTLA-8 (B18) expressed *in vivo* was examined by construction of a recombinant adenovirus vector.

The B18 cDNA in the expression plasmid Adori 2-12 B18 was driven by the cytomegalovirus(CMV) immediate early promoter and enhancer.

20

The Adori 2-12 vector was created by addition of an SV40 origin and enhancer to a known adenovirus vector (Barr et al., Gene Therapy 1:51 (1994); Davidson et al., Nature Genetics 3:219 (1993)). The HindIII/BamHI fragment encoding the SV40 origin and enhancer was isolated from the pMT2 mammalian expression vector, blunted with Klenow and cloned into the NaiI site (blunted with Klenow) of the Ad5 expression vector.

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The vector was derived by digesting pNOT-B18 cDNA with *Sal*II, filling in the 5' overhang with Klenow to generate a blunt end and digesting with *Eco*RI to isolate the B18 cDNA. The blunted- *Eco*RI B18 fragment was inserted into the restriction sites *Eco*RV-*Eco*RI of the adenovirus vector Adori 2-12. The CMV-B18 expression cassette was located downstream of the SV40 origin and enhancer, and 0-1 map units of the left hand end of the adenovirus type 5(Ad5). The SV40 splice donor and

acceptor were located between the CMV promoter and B18 cDNA. Following the insert was SV40 poly A site, 9-16 map units of Ad5 and the puc 19 origin.

A recombinant adenovirus was generated by homologous recombination in 293 cells. *Ascl*I linearized Adori 2-12 B18 and *Cla*I digested AdCMVlacZ were introduced 5 into the 293 cells using lipofectamine. Recombinant adenovirus virus was isolated and amplified on 293 cells. The virus was released from infected 293 cells by three cycles of freeze-thawing. The virus was further purified by two cesium chloride centrifugation gradients and dialyzed against PBS 4°C. Following dialysis of the virus glycerol was added to a concentration of 10 % and the virus was stored at - 70 °C until 10 use. The virus was characterized by expression of the transgene, plaque forming units on 293 cells, particles/ml and Southern analysis of the virus.

A single dose of 5×10^{10} particles of recombinant adenovirus encoding B18 was injected into the tail vein of male C57/bl6 mice, age 7-8 weeks. Control mice received an adenovirus encoding B-galactosidase. Four mice from each experimental 15 group were killed on day 7 and 14. Blood was collected and automated hematologic analysis was performed using a Baker 9000. Differential counts were performed on blood smears. Tissue was harvested, fixed in formalin, and stained with hematoxylin and eosin for histopathology. In the first set of experiments, serum and tissues were analyzed 7 and 14 days post injection. A slight increase in peripheral platelet counts 20 were observed. The animals that received B18 exhibited a slight increase in spleen size. Macroscopic analysis of the spleen showed an increase in splenic extramedullary hematopoiesis on day 7 compared to the control. These results showed a hematopoietic growth activity associated with B18.

In a second set of experiments 5×10^{10} particles of recombinant adenovirus 25 encoding B18 were injected into the tail vein of male C57/bl6 mice, age 17-18 weeks. Control mice received an adenovirus encoding B-galactosidase. Blood samples were collected via retro-orbital sinus on days 2, 5, 7, 10, 14, and 21. The hematologic analyses were performed on the Baker 9000 automated cell counter with murine-specific settings. Analyses included WBC, RBC, HCT and PLT counts. Blood smears 30 were prepared and stained with Wright-Geimsa for WBC differentials based on a 100 cellcount. Reticulocytes and reticulated platelets were quantitated using flow

cytometry. Four mice from each group were killed on days 7, 14, and 21. In addition to peripheral blood analysis, serum was collected via cardiac puncture for quantitation of systemic IL-6 using a commercial kit (Endogen). Spleen and liver were collected for histopathology, spleen and bone marrow hematopoietic progenitors were quantitated, 5 and bone marrow smears were prepared and stained with Wright-Geimsa for cell counts.

10 Administration of adenovirus encoding B18 resulted in a marked increase in peripheral blood neutrophils and WBC (Fig. 4). Maximum increases in neutrophils were observed at day 5 and day 7. The control mice showed little difference at day 5 and day 7. Peripheral blood neutrophils were similar in the control mice and mice that received B18 at day 21. In both the B18 and control groups an increase in white blood cells was also observed. The mice that received B18 had a greater increase in WBC between day 2 and day 7. By Day 21 a more pronounced increase was observed in the B-gal group. No other changes in cellular chemistries were observed (Table I).

15 Bone marrow cellularity was calculated from pooled femurs in each group (Table III). No significant differences were observed in either group. No significant changes were observed in bone marrow hematopoietic progenitors from day 7, 14, and 21. The CFU-GM, BFU-E and CFU-MEG in the B18 mice were similar to the B-gal control (Table II).

20 Administration of the adenovirus encoding B18 resulted in an increase in CFU-GM (myeloid) and BFU-E (erythroid) progenitors in the spleen compared to animals that received the B-gal virus on day 7. The increase in progenitors in the B18 mice was 11-fold in CFU-GM and a 52-fold in BFU-E (Table II). There was a 2-fold increase in CFU-MEG at day 7 for the B18 mice. By day 21 no significant differences 25 were observed in splenic CFU-MEG or BFU-E between the groups (Table II). A 3-fold decrease in CFU-GM was observed in mice that received adenovirus encoding B18. A slight increase in spleen size at day 7 was observed in the B18 group. This is consistent with an increase in splenic cellularity. By day 14 and day 21 spleen weights were similar to the control group (Table III). Macroscopic analysis of the 30 spleen showed an increase in splenic extramedullary hematopoiesis of the B18 mice on day 7 compared to the control.

The bone marrow myeloid: erythroid ratios (Table IV) suggest a granulocytic hyperplasia with a possible erythroid hypoplasia in mice that received adenovirus B18 on day 7. By day 21 the ratio in the B-gal group was higher. No changes were observed in the IL6 serum levels.

5 These results show a hematopoietic activity associated with the administration of adenovirus encoding B18 (human CTLA-8). Increases in neutrophils and white blood cells were observed at day 7 in animals that received B18 adenovirus. The data showed that B18 resulted in increase in splenic CFU-GM and BFU-E 7 days post administration compared to the control animals. Splenic extramedullary 10 hematopoiesis on day 7 support that B18 exhibits a hematopoietic growth activity. These data suggest that B18 may mobilize early hematopoietic precursors.

Table I: Peripheral hematology for day 2, 5, 7, 10, 14, and 21.

Study A54-4D...B18 (Platelets) Day 2...4-25-96.														
Group A	WBC x10 ³ /uL	Neuts %	ANC % x10 ³ /uL	Lymphs % x10 ³ /uL	ALC % x10 ³ /uL	Eos %	Monos %	RBC x10 ⁶ /uL	Retics %	Abs Retics x10 ⁶ /uL	HCT %	PLT x10 ³ /uL	RPT %	Abs RPT x10 ⁻³ /uL
B-Gal #1	5.4	40	2.16	54	2.92	0	6	10.88	3.65	0.40	48.0	836	11.94	99.82
B-Gal #2	7.4	25	1.85	65	4.81	3	7	12.34	2.04	0.25	56.6	900	10.10	90.90
B-Gal #3	6.8	40	2.72	52	3.54	2	6	11.26	3.26	0.37	51.6	894	9.77	87.34
B-Gal #4	8.8	23	2.02	64	5.63	1	12	12.00	2.55	0.31	54.8	840	10.63	89.29
AVG	7.1	32.0	2.19	58.8	4.22	1.5	7.8	11.62	2.86	0.33	52.8	868	10.61	91.84
SEM	0.7	4.6	0.19	3.4	0.61	0.6	1.4	0.33	0.36	0.03	1.9	17	0.48	2.76
B18 #1	11.4	59	6.73	31	3.53	1	9	11.16	4.88	0.54	52.4	1242	14.92	185.31
B18 #2	9.2	30	2.76	62	5.70	3	5	10.14	3.97	0.40	48.0	632	10.90	68.89
B18 #3	5.0	51	2.55	40	2.00	0	9	11.16	3.23	0.36	51.2	832	11.18	93.02
B18 #4	6.4	41	2.62	55	3.52	0	4	10.80	3.09	0.33	49.2	904	17.31	156.48
AVG	8.0	45.3	3.67	47.0	3.69	1.0	6.8	10.82	3.79	0.41	50.2	903	13.58	125.92
SEM	1.4	6.3	1.02	7.0	0.76	0.7	1.3	0.24	0.41	0.05	1.0	127	1.55	27.07
Study A54-4B...B18 (Platelets) Day 5...4-28-96.														
Group B	WBC x10 ³ /uL	Neuts %	ANC % x10 ³ /uL	Lymphs % x10 ³ /uL	ALC % x10 ³ /uL	Eos %	Monos %	RBC x10 ⁶ /uL	Retics %	Abs Retics x10 ⁶ /uL	HCT %	PLT x10 ³ /uL	RPT %	Abs RPT x10 ⁻³ /uL
B-Gal #1	7.6	14	1.06	78	5.93	3	5	11.26	5.25	0.59	52.4	1082	15.51	167.82
B-Gal #2	10.6	20	2.12	78	6.27	1	1	10.72	4.71	0.50	49.4	994	17.37	172.66
B-Gal #3	8.8	18	-1.51	69	6.07	2	11	11.12	3.40	0.38	51.2	916	9.55	87.48
B-Gal #4	10.8	38	4.10	58	6.26	0	4	10.22	6.21	0.63	47.0	1092	13.93	152.12
AVG	9.5	22.5	2.20	70.8	6.63	1.5	5.3	10.83	4.89	0.53	50.0	1021	14.09	145.02
SEM	0.8	5.3	0.67	4.8	0.55	0.6	2.1	0.23	0.59	0.06	1.2	41	1.57	19.57
B18 #1	14.8	18	2.66	71	10.51	1	10	12.66	2.31	0.29	57.0	1204	7.57	91.14
B18 #2	14.2	37	5.25	53	7.53	2	8	9.80	3.32	0.33	44.6	888	14.33	127.25
B18 #3	12.8	30	3.84	59	7.55	1	10	12.12	4.12	0.50	55.6	1134	10.15	115.10
B18 #4	16.0	58	9.28	37	5.92	0	5	11.04	3.93	0.43	50.8	1166	15.75	183.65
AVG	14.5	35.8	5.26	55.0	7.88	1.0	8.3	11.41	3.42	0.39	52.0	1098	11.95	129.28
SEM	0.7	8.4	1.44	7.1	0.96	0.4	1.2	0.63	0.41	0.05	2.8	71	1.88	19.51
Study A54-4B...B18 (Platelets) Day 7...4-30-96.														
Group C	WBC x10 ³ /uL	Neuts %	ANC % x10 ³ /uL	Lymphs % x10 ³ /uL	ALC % x10 ³ /uL	Eos %	Monos %	RBC x10 ⁶ /uL	Retics %	Abs Retics x10 ⁶ /uL	HCT %	PLT x10 ³ /uL	RPT %	Abs RPT x10 ⁻³ /uL
B-Gal #1	15.2	14	2.13	69	10.49	1	16	11.04	3.54	0.39	50.8	862	12.46	107.41
B-Gal #2	14.0	12	1.68	81	11.34	0	7	11.38	5.05	0.57	52.6	1104	14.91	164.61
B-Gal #3	14.8	14	2.07	73	10.80	1	12	10.92	5.42	0.59	49.6	952	11.49	109.38
AVG	14.7	13.3	1.96	74.3	10.88	0.7	11.7	11.11	4.57	0.52	51.0	973	12.35	127.13
SEM	0.4	0.7	0.14	3.5	0.25	0.3	2.6	0.14	0.58	0.06	0.9	71	1.02	18.75
B18 #1	19.4	33	6.40	62	12.03	0	5	10.14	2.93	0.30	45.2	864	12.80	110.59
B18 #2	25.4	39	9.91	53	13.46	0	8	9.46	6.05	0.57	43.6	1288	12.49	160.87
B18 #3	23.6	44	10.38	50	11.80	0	6	9.74	5.17	0.50	44.4	1076	15.41	165.81
B18 #4	12.8	15	1.92	75	9.60	0	10	9.54	6.26	0.60	43.4	1136	15.88	180.40
AVG	20.3	32.8	7.15	60.0	11.72	0.0	7.3	9.72	5.10	0.49	44.2	1091	14.15	154.42
SEM	2.5	6.3	1.96	5.6	0.80	0.0	1.1	0.15	0.76	0.07	0.4	88	0.87	15.19
Study A54-4B...B18 (Platelets) Day 10...5-3-96.														
Group A	WBC x10 ³ /uL	Neuts %	ANC % x10 ³ /uL	Lymphs % x10 ³ /uL	ALC % x10 ³ /uL	Eos %	Monos %	RBC x10 ⁶ /uL	Retics %	Abs Retics x10 ⁶ /uL	HCT %	PLT x10 ³ /uL	RPT %	Abs RPT x10 ⁻³ /uL
B-Gal #1	18.6	17	3.15	69	12.83	3	11	10.22	12.41	1.27	46.8	1460	16.20	236.52
B-Gal #2	13.2	16	2.11	79	10.43	1	4	10.48	6.00	0.63	48.8	1128	14.48	163.33
B-Gal #3	19.6	16	3.14	74	14.50	0	10	10.72	6.25	0.67	49.4	1338	16.58	221.84
B-Gal #4	18.6	21	3.91	72	13.39	3	4	10.44	7.59	0.79	48.4	1003	14.35	153.06
AVG	17.5	17.5	3.08	73.5	12.79	1.8	7.3	10.47	8.06	0.84	48.4	1249	15.40	193.74
SEM	1.5	1.2	0.37	2.1	0.86	0.8	1.9	0.10	1.49	0.15	0.6	91	0.58	20.78
B18 #1	14.2	33	4.69	56	7.95	5	6	6.70	11.57	1.04	39.2	1760	14.49	255.02
B18 #2	17.6	35	6.16	57	10.03	1	7	9.04	9.48	0.86	42.0	1104	18.88	206.44
B18 #3	16.2	39	6.32	57	9.23	1	3	4.74	16.77	0.79	22.4	894	23.19	260.96
B18 #4	14.2	25	3.55	66	9.37	1	8	9.30	9.93	0.92	42.0	1416	16.81	238.03
AVG	15.6	33.0	6.18	59.0	9.15	2.0	6.0	7.95	12.04	0.90	36.4	1294	19.84	240.61
SEM	0.8	2.9	0.66	2.3	0.43	1.0	1.1	1.05	1.57	0.05	4.7	189	3.24	11.77
Study A54-4B...B18 (Platelets) Day 14...5-7-96.														
Group B	WBC x10 ³ /uL	Neuts %	ANC % x10 ³ /uL	Lymphs % x10 ³ /uL	ALC % x10 ³ /uL	Eos %	Monos %	RBC x10 ⁶ /uL	Retics %	Abs Retics x10 ⁶ /uL	HCT %	PLT x10 ³ /uL	RPT %	Abs RPT x10 ⁻³ /uL
B-Gal #1	17.8	18	3.20	74	13.17	0	8	10.66	5.97	0.65	50.8	1360	11.03	150.01
B-Gal #2	20.4	26	5.30	66	13.46	1	7	10.92	7.07	0.77	50.8	1616	8.18	132.19
B-Gal #3	16.0	7	1.12	90	14.40	1	3	11.36	6.41	0.73	52.8	1298	7.36	95.53
B-Gal #4	18.0	36	6.48	57	10.26	1	6	9.30	7.62	0.71	43.0	1672	10.05	168.04
AVG	18.1	21.8	4.03	71.8	12.82	0.8	6.0	10.61	6.77	0.71	49.4	1487	9.16	136.44
SEM	0.9	6.1	1.18	7.0	0.89	0.3	1.1	0.45	0.36	0.03	2.2	93	0.84	15.44
B18 #1	15.4	9	1.39	81	12.47	1	9	10.62	5.74	0.61	48.2	1262	9.51	120.02
B18 #2	15.4	31	4.77	58	8.03	2	9	9.76	10.33	1.01	44.6	1092	14.29	156.05
B18 #3	13.4	42	5.63	39	5.22	0	19	10.34	4.99	0.52	46.6	1376	15.79	217.27
B18 #4	11.6	57	6.61	34	3.94	2	7	9.38	8.57	0.52	43.0	1092	16.66	161.93
AVG	14.0	34.8	4.60	53.0	7.64	1.3	11.0	10.03	6.66	0.66	45.6	1206	14.06	168.82
SEM	0.9	10.1	1.14	10.7	1.93	0.5	2.7	0.26	1.23	0.12	1.1	70	1.59	20.54
Study A54-4B...B18 (Platelets) Day 21...5-14-96.														
Group A	WBC x10 ³ /uL	Neuts %	ANC % x10 ³ /uL	Lymphs % x10 ³ /uL	ALC % x10 ³ /uL	Eos %	Monos %	RBC x10 ⁶ /uL	Retics %	Abs Retics x10 ⁶ /uL	HCT %	PLT x10 ³ /uL	RPT %	Abs RPT x10 ⁻³ /uL
B-Gal #1	25.4	23	5.84	67	17.02	0	10	9.22	8.15	0.75	42.8	1776	9.61	170.67
B-Gal #2	19.6	19	3.72	69	13.52	0	12	9.50	9.95	0.95	44.4	1662	9.44	156.89
B-Gal #3	27.6	11	3.04	82	22.63	3	4	9.74	8.84	0.86	45.8	1684	11.45	192.82
B-Gal #4	28.0	13	3.64	83	23.24	0</td								

Table II: Bone marrow and Splenic Hematopoietic Progenitors

	CFU-MEG		CFU-GM		BFU-E	
	B-Gal	B18	B-Gal	B18	B-Gal	B18
5	Day 7	16.0 ± 3.5	15.7 ± 3.1	307 ± 117	241 ± 78	51 ± 19
	Day 14	10.7 ± 2.3	15.3 ± 1.2	233 ± 15	373 ± 35	30 ± 10
	Day 21	5.7 ± 0.6	6.7 ± 3.1	170 ± 17	160 ± 27	40 ± 10
	Spleen**					
10	Day 7	9.3 ± 1.6	19.5 ± 1.5	27 ± 3	298 ± 6	1.3 ± 1.2
	Day 14	9.7 ± 0.6	12.7 ± 0.6	267 ± 32	197 ± 21	33 ± 6
	Day 21	17.0 ± 1.0	19.3 ± 2.5	187 ± 6	73 ± 15	23 ± 6

Hematopoietic precursors were determined from pooled spleen and bone marrow samples from four animals in each group. For quantitation of CFU-GM and BFU-E, either 1×10^4 bone marrow cells or 1×10^5 spleen cells were added to complete alpha methylcellulose medium (0.9% methylcellulose in alpha medium, 30% fetal bovine serum, 1% bovine serum albumin, 10-4M 2-mercaptoethanol, 2 mM L-glutamine, 2% murine spleen cell conditioned medium, and 3 U/mL erythropoietin) and aliquoted into 35 mm tissue culture dishes in a final volume of 1.0 mL. Cultures were incubated for 7 days at 37°C, and 5% CO₂. Microscopic colonies were defined as clusters of 50 or more cells. For quantitation of CFU-MEG, either 1×10^5 bone marrow cells or 1×10^6 spleen cells were added to complete alpha methylcellulose medium and incubated as described above. Megakaryocyte colonies were defined as a group of 3 or more cells.

*Bone marrow progenitors are represented as mean ± sd number of colonies per 10^5 cells.

**Spleen progenitors are represented as mean ± sd number of colonies per 10^6 cells.

Table III: Spleen Weights and Femur Cellularity

Spleen Wt. (Mg)	B-Gal	B18		Femur Cellularity (x10 ⁶)	B-Gal	B18
Day 7	187 ± 19	224 ± 29		Day 7	28	23
Day 14	175 ± 13	170 ± 10		Day 14	28	27
Day 21	174 ± 21	151 ± 27		Day 21	28	26

5 Spleen weights were determined at time of sacrifice are represented as means ± sd from four animals.

10 Table IV: Bone Marrow Myeloid:Erythroid Ratios

Group	Mouse #	Day 7	Day 14	Day 21
B-gal	1	1.43	2.12	5.78
	2	0.91	2.46	5.83
	3	1.62	1.03	3.66
	4		5.44	4.82
AVG		1.32	2.76	5.02
SD		0.37	0.37	1.89
B-gal	4	5.59	2.01	2.02
	2	6.51	1.25	2.13
	3	5.49	1.58	1.81
	4	0.50	2.51	2.92
AVG		4.52	1.86	2.22
SD		1.29	2.72	0.56

15 20 25 All entries represent the number of myeloid cells per 1 erythroid cell. Normal mouse ratios are approximately 1:1 to 2:1.

Example 5

30 Additional Experiments Relating to

Hematopoietic Activity of Human CTLA-8

B18 (human CTLA-8) was tested for the ability to induce production of factors having hematopoietic activity in a factor-dependent cell proliferation assay using the

human erythroleukemic cell line, TF-1 (Kitamura et al., J. Cell Physiol. 140:323 (1989)). The cells were initially grown in the presence of rhGMCSF (100 U/ml). The cells were fed three days prior to setting up the assay. The assay conditions were as follows:

5	cells/well	5000/200μl
	incubation time	3 days
	pulse time	4 hours
	amount of tritiated thymidine	0.5μCi/well
	counting time	1 minute
10	replicates	2

B18 alone, conditioned medium (CM) from B18 induced HS-5 cells were assayed. Buffer alone, CM from HS-5 cells induced with buffer and CM from uninduced HS-5 cells were assayed as controls. Results are shown in Fig. 5. B18 (human CTLA-8) demonstrated an ability to induce production of factors which induced TF-1 proliferation. This activity was substantially eliminated by the addition of anti-GMCSF antibodies. These data demonstrate that human CTLA-8 (B18) is able to induce hematopoiesis. Particularly, without being bound by any theory, it appears that human CTLA-8 (B18) induces production of GM-CSF and/or IL-3.

20

Example 6

Ability of Human CTLA-8 to Induce Production of IL-6 and IL-8

MRC5 cells were incubated in the presence of human CTLA-8 (B18) and production of IL-6 and IL-8 were measured. Herpes CTLA-8 (IL-17) was used as a positive control. Applicants' human CTLA-8 (B18) demonstrated titratable production of both IL-6 and IL-8 (see Figs. 6 and 7).

All patent and literature references cited herein are incorporated by reference as if fully set forth.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Human CTLA-8 and Uses of CTLA-8-Related Proteins

(iii) NUMBER OF SEQUENCES: 9

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(B) REGISTRATION NUMBER: 32,724
(C) REFERENCE/DOCKET NUMBER: GI5262

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 813 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 56..544

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGAAGATAC ATT CACAGAA AGAGCTTCCT GCACAAAGTA AGCCACCAGC GCAAC ATG Met 1	58
ACA GTG AAG ACC CTG CAT GGC CCA GCC ATG GTC AAG TAC TTG CTG CTG Thr Val Lys Thr Leu His Gly Pro Ala Met Val Lys Tyr Leu Leu Leu 5 10 15	106
TCG ATA TTG GGG CTT GCC TTT CTG AGT GAG GCG GCA GCT CGG AAA ATC Ser Ile Leu Gly Leu Ala Phe Leu Ser Glu Ala Ala Arg Lys Ile 20 25 30	154
CCC AAA GTA GGA CAT ACT TTT TTC CAA AAG CCT GAG AGT TGC CCG CCT Pro Lys Val Gly His Thr Phe Phe Gln Lys Pro Glu Ser Cys Pro Pro 35 40 45	202
GTG CCA GGA GGT AGT ATG AAG CTT GAC ATT GGC ATC ATC AAT GAA AAC Val Pro Gly Gly Ser Met Lys Leu Asp Ile Gly Ile Ile Asn Glu Asn 50 55 60 65	250
CAG CGC GTT TCC ATG TCA CGT AAC ATC GAG AGC CGC TCC ACC TCC CCC Gln Arg Val Ser Met Ser Arg Asn Ile Glu Ser Arg Ser Thr Ser Pro 70 75 80	298
TGG AAT TAC ACT GTC ACT TGG GAC CCC AAC CGG TAC CCC TCG GAA GTT Trp Asn Tyr Thr Val Thr Trp Asp Pro Asn Arg Tyr Pro Ser Glu Val 85 90 95	346
GTA CAG GCC CAG TGT AGG AAC TTG GGC TGC ATC AAT GCT CAA GGA AAG Val Gln Ala Gln Cys Arg Asn Leu Gly Cys Ile Asn Ala Gln Gly Lys 100 105 110	394
GAA GAC ATC TCC ATG AAT TCC GTT CCC ATC CAG CAA GAG ACC CTG GTC Glu Asp Ile Ser Met Asn Ser Val Pro Ile Gln Gln Glu Thr Leu Val 115 120 125	442
GTC CGG AGG AAG CAC CAA GGC TGC TCT GTT TCT TTC CAG TTG GAG AAG Val Arg Arg Lys His Gln Gly Cys Ser Val Ser Phe Gln Leu Glu Lys 130 135 140 145	490
G TG CTG GTG ACT GTT GGC TGC ACC TGC GTC ACC CCT GTC ATC CAC CAT Val Leu Val Thr Val Gly Cys Thr Cys Val Thr Pro Val Ile His His 150 155 160	538
G TG CAG TAAGAGGTGC ATATCCACTC AGCTGAAGAA GCTGTAGAAA TGCCACTCCT Val Gln	594
TACCCAGTGC TCTGCAACAA GTCCTGTCTG ACCCCCCATT CCCTCCACTT CACAGGACTC TTAATAAGAC CTGCACGGAT GGAAACAGAA AATATTCA CA ATGTATGTGT GTATGTACTA CACTTTATAT TTGATATCTA AAATGTTAGG AGAAAAATTA ATATATTCA TGCTAATATA ATAAAGTATT AATAATTAA AAATAAAAAA AAAAAAAA	654 714 774 813

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 163 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Val Lys Thr Leu His Gly Pro Ala Met Val Lys Tyr Leu Leu
 1 5 10 15

Leu Ser Ile Leu Gly Leu Ala Phe Leu Ser Glu Ala Ala Ala Arg Lys
 20 25 30

Ile Pro Lys Val Gly His Thr Phe Phe Gln Lys Pro Glu Ser Cys Pro
 35 40 45

Pro Val Pro Gly Gly Ser Met Lys Leu Asp Ile Gly Ile Ile Asn Glu
 50 55 60

Asn Gln Arg Val Ser Met Ser Arg Asn Ile Glu Ser Arg Ser Thr Ser
 65 70 75 80

Pro Trp Asn Tyr Thr Val Thr Trp Asp Pro Asn Arg Tyr Pro Ser Glu
 85 90 95

Val Val Gln Ala Gln Cys Arg Asn Leu Gly Cys Ile Asn Ala Gln Gly
 100 105 110

Lys Glu Asp Ile Ser Met Asn Ser Val Pro Ile Gln Gln Glu Thr Leu
 115 120 125

Val Val Arg Arg Lys His Gln Gly Cys Ser Val Ser Phe Gln Leu Glu
 130 135 140

Lys Val Leu Val Thr Val Gly Cys Thr Cys Val Thr Pro Val Ile His
 145 150 155 160

His Val Gln

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 461 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 6..455

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCACC ATG TGC CTG ATG CTG TTG CTG CTA CTG AAC CTG GAG GCT ACA	47
Met Cys Leu Met Leu Leu Leu Leu Asn Leu Glu Ala Thr	
1 5 10	
GTG AAG GCA GCG GTA CTC ATC CCT CAA AGT TCA GTG TGT CCA AAC GCC	95
Val Lys Ala Ala Val Leu Ile Pro Gln Ser Ser Val Cys Pro Asn Ala	
15 20 25 30	
GAG GCC AAT AAC TTT CTC CAG AAC GTG AAG GTC AAC CTG AAA GTC ATC	143
Glu Ala Asn Asn Phe Leu Gln Asn Val Lys Val Asn Leu Lys Val Ile	
35 40 45	

AAC TCC CTT AGC TCA AAA GCG AGC TCG AGA AGG CCC TCA GAT TAC CTC Asn Ser Leu Ser Ser Lys Ala Ser Ser Arg Arg Pro Ser Asp Tyr Leu 50 55 60	191
AAC CGT TCC ACT TCA CCC TGG ACT CTG AGC CGC AAT GAG GAC CCT GAT Asn Arg Ser Thr Ser Pro Trp Thr Leu Ser Arg Asn Glu Asp Pro Asp 65 70 75	239
AGA TAT CCT TCT GTG ATC TGG GAG GCA CAG TGC CGC CAC CAG CGC TGT Arg Tyr Pro Ser Val Ile Trp Glu Ala Gln Cys Arg His Gln Arg Cys 80 85 90	287
GTC AAC GCT GAG GGG AAG TTG GAC CAC CAC ATG AAT TCT GTT CTC ATC Val Asn Ala Glu Gly Lys Leu Asp His His Met Asn Ser Val Leu Ile 95 100 105 110	335
CAG CAA GAG ATA CTA GTC CTG AAG AGG GAG CCT GAG AAG TGC CCC TTC Gln Gln Glu Ile Leu Val Leu Lys Arg Glu Pro Glu Lys Cys Pro Phe 115 120 125	383
ACT TTC CGG GTG GAG AAG ATG CTG GTG GGC GTG GGC TGC ACC TGC GTT Thr Phe Arg Val Glu Lys Met Leu Val Gly Val Gly Cys Thr Cys Val 130 135 140	431
TCC TCT ATT GTC CGC CAT GCG TCC TAATAA Ser Ser Ile Val Arg His Ala Ser 145 150	461

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 150 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Cys Leu Met Leu Leu Leu Leu Asn Leu Glu Ala Thr Val Lys 1 5 10 15	
Ala Ala Val Leu Ile Pro Gln Ser Ser Val Cys Pro Asn Ala Glu Ala 20 25 30	
Asn Asn Phe Leu Gln Asn Val Lys Val Asn Leu Lys Val Ile Asn Ser 35 40 45	
Leu Ser Ser Lys Ala Ser Ser Arg Arg Pro Ser Asp Tyr Leu Asn Arg 50 55 60	
Ser Thr Ser Pro Trp Thr Leu Ser Arg Asn Glu Asp Pro Asp Arg Tyr 65 70 75 80	
Pro Ser Val Ile Trp Glu Ala Gln Cys Arg His Gln Arg Cys Val Asn 85 90 95	
Ala Glu Gly Lys Leu Asp His His Met Asn Ser Val Leu Ile Gln Gln 100 105 110	
Glu Ile Leu Val Leu Lys Arg Glu Pro Glu Lys Cys Pro Phe Thr Phe 115 120 125	
Arg Val Glu Lys Met Leu Val Gly Val Gly Cys Thr Cys Val Ser Ser 130 135 140	

Ile Val Arg His Ala Ser
145 150

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 459 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..453

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG ACA TTT AGA ATG ACT TCA CTT GTG TTA CTT CTG CTG CTG AGC ATA Met Thr Phe Arg Met Thr Ser Leu Val Leu Leu Leu Leu Ser Ile 1 5 10 15	48
GAT TGT ATA GTA AAG TCA GAA ATA ACT AGT GCA CAA ACC CCA AGA TGC Asp Cys Ile Val Lys Ser Glu Ile Thr Ser Ala Gln Thr Pro Arg Cys 20 25 30	96
TTA GCT GCT AAC AAT AGC TTT CCA CGG TCT GTG ATG GTT ACT TTG AGC Leu Ala Ala Asn Asn Ser Phe Pro Arg Ser Val Met Val Thr Leu Ser 35 40 45	144
ATC CGT AAC TGG AAT ACC AGT TCT AAA AGG GCT TCA GAC TAC TAC AAT Ile Arg Asn Trp Asn Thr Ser Ser Lys Arg Ala Ser Asp Tyr Tyr Asn 50 55 60	192
AGA TCT ACG TCT CCT TGG ACT CTC CAT CGC AAT GAA GAT CAA GAT AGA Arg Ser Thr Ser Pro Trp Thr Leu His Arg Asn Glu Asp Gln Asp Arg 65 70 75 80	240
TAT CCC TCT GTG ATT TGG GAA GCA AAG TGT CGC TAC TTA GGA TGT GTT Tyr Pro Ser Val Ile Trp Glu Ala Lys Cys Arg Tyr Leu Gly Cys Val 85 90 95	288
AAT GCT GAT GGG AAT GTA GAC TAC CAC ATG AAC TCA GTC CCT ATC CAA Asn Ala Asp Gly Asn Val Asp Tyr His Met Asn Ser Val Pro Ile Gln 100 105 110	336
CAA GAG ATT CTA GTG GTG CGC AAA GGG CAT CAA CCC TGC CCT AAT TCA Gln Glu Ile Leu Val Val Arg Lys Gly His Gln Pro Cys Pro Asn Ser 115 120 125	384
TTT AGG CTA GAG AAG ATG CTA GTG ACT GTA GGC TGC ACA TGC GTT ACT Phe Arg Leu Glu Lys Met Leu Val Thr Val Gly Cys Thr Cys Val Thr 130 135 140	432
CCC ATT GTT CAC AAT GTA GAC TAAAAG Pro Ile Val His Asn Val Asp 145 150	459

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 151 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Thr	Phe	Arg	Met	Thr	Ser	Leu	Val	Leu	Leu	Leu	Leu	Ser	Ile	
1				5					10					15	
Asp	Cys	Ile	Val	Lys	Ser	Glu	Ile	Thr	Ser	Ala	Gln	Thr	Pro	Arg	Cys
				20				25					30		
Leu	Ala	Ala	Asn	Asn	Ser	Phe	Pro	Arg	Ser	Val	Met	Val	Thr	Leu	Ser
				35				40					45		
Ile	Arg	Asn	Trp	Asn	Thr	Ser	Ser	Lys	Arg	Ala	Ser	Asp	Tyr	Tyr	Asn
				50			55					60			
Arg	Ser	Thr	Ser	Pro	Trp	Thr	Leu	His	Arg	Asn	Glu	Asp	Gln	Asp	Arg
				65			70			75			80		
Tyr	Pro	Ser	Val	Ile	Trp	Glu	Ala	Lys	Cys	Arg	Tyr	Leu	Gly	Cys	Val
				85				90					95		
Asn	Ala	Asp	Gly	Asn	Val	Asp	Tyr	His	Met	Asn	Ser	Val	Pro	Ile	Gln
				100				105					110		
Gln	Glu	Ile	Leu	Val	Val	Arg	Lys	Gly	His	Gln	Pro	Cys	Pro	Asn	Ser
				115			120					125			
Phe	Arg	Leu	Glu	Lys	Met	Leu	Val	Thr	Val	Gly	Cys	Thr	Cys	Val	Thr
				130			135					140			
Pro	Ile	Val	His	Asn	Val	Asp									
				145			150								

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACAGGCATA CACAGGAAGA TACATTCA

28

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCTTGCTGGA TGGGAACGGA ATTCA

25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATACATTACAGAAGAGCTT CCTGCACA

28

5 What is claimed is:

1. An isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 146 to nucleotide 544;

10 (b) a nucleotide sequence capable of hybridizing to a nucleic acid sequence specified in (a);

 (c) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code; and

 (d) an allelic variant of the nucleotide sequence specified in (a).

15

2. The polynucleotide of claim 1 wherein said nucleotide sequence encodes a protein having CTLA-8 activity.

20 3. The polynucleotide of claim 1 wherein said nucleotide sequence is operably linked to an expression control sequence.

4. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 55 to nucleotide 544.

25 5. A host cell transformed with the polynucleotide of claim 3.

6. The host cell of claim 5, wherein said cell is a mammalian cell.

7. A process for producing a human CTLA-8 protein, said process comprising:

30 (a) growing a culture of the host cell of claim 5 in a suitable culture medium; and

 (b) purifying the human CTLA-8 protein from the culture.

5 8. An isolated human CTLA-8 protein comprising an amino acid sequence selected from the group consisting of:

- (a) the amino acid sequence of SEQ ID NO:2;
- (b) the amino acid sequence of SEQ ID NO:2 from amino acids 11 to 163;
- (c) the amino acid sequence of SEQ ID NO:2 from amino acids 29 to 163;
- 10 (d) the amino acid sequence of SEQ ID NO:2 from amino acids 31 to 163; and
- (e) fragments of (a), (b), (c) or (d) having CTLA-8 activity.

9. The protein of claim 8 comprising the amino acid sequence of SEQ ID NO:2.

15 10. The protein of claim 8 comprising the sequence from amino acid 29 to 163 of SEQ ID NO:2.

11. A pharmaceutical composition comprising a human CTLA-8 protein of claim 8 and a pharmaceutically acceptable carrier.

20 12. A human CTLA-8 protein produced according to the process of claim 7.

13. A composition comprising an antibody which specifically reacts with a human CTLA-8 protein of claim 8.

25 14. A method of treating a mammalian subject comprising administering a therapeutically effective amount of a composition of claim 11.

30 15. A method of treating a mammalian subject comprising administering a therapeutically effective amount of a composition comprising a pharmaceutically acceptable carrier and a protein comprising an amino acid sequence selected from the group consisting of:

- (a) the amino acid sequence of SEQ ID NO:4;

5 (b) the amino acid sequence of SEQ ID NO:4 from amino acids 18 to 150; and
 (c) fragments of (a) or (b) having CTLA-8 activity.

16. The method of claim 15 wherein said protein comprises the amino acid sequence
of SEQ ID NO:4.

10

17. The method of claim 15 wherein said protein comprises the amino acid sequence
of SEQ ID NO:4 from amino acids 18 to 150.

15

18. A method of treating a mammalian subject administering a therapeutically effective
amount of a composition comprising a pharmaceutically acceptable carrier and a protein
comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:6;
 (b) the amino acid sequence of SEQ ID NO:6 from amino acids 19 to 151; and
 (c) fragments of (a) or (b) having CTLA-8 activity.

20

19. The method of claim 18 wherein said protein comprises the amino acid sequence
of SEQ ID NO:6.

25

20. The method of claim 18 wherein said protein comprises the amino acid sequence
of SEQ ID NO:6 from amino acids 19 to 151.

21. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1
from nucleotide 86 to nucleotide 544.

30

22. The protein of claim 8 comprising the sequence from amino acid 11 to 163 of SEQ
ID NO:2.

5 23. A method of treating a mammalian subject comprising administering a
therapeutically effective amount of a composition comprising a pharmaceutically
acceptable carrier and IL-17 or an active fragment thereof.

10 24. The method of claim 14, 15, 18 or 23 wherein said subject is treated to produce an
effect selected from the group consisting of inhibition of angiogenesis, inhibition of growth
or proliferation of vascular endothelial cells, inhibition of tumor growth, inhibition of
angiogenesis-dependent tissue growth, proliferation of myeloid cells or progenitors,
proliferation of erythroid cells or progenitors, proliferation of lymphoid cells or progenitors,
induction of IFN γ production, induction of IL-3 production and induction of GM-CSF
15 production.

25. The composition of claim 3 wherein said polynucleotide is contained in a vector
suitable for *in vivo* expression in a mammalian subject.

20 26. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1
from nucleotide 139 to nucleotide 544.

27. The protein of claim 8 comprising the sequence from amino acid 31 to 163 of SEQ
ID NO:2.

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Hsvie_2	- - - - -	V[K] S E[I] T S[A] Q - T P R C L - A A N N S[F] P R S V M[Y] T L[S] I[R] N - - W[N] T S S[K] R[A] S D[Y] N[R] S T S[P]	51
Musctla8	- - - - -	S S V C P N A E A N N[F] L Q N V K Y N L K V I N S L S S[K] A S S R[R] P S D[Y] L N[R] S T S[P]	51
B18_F1	A A R K[I] P[K] V G H[T] F F	K P E S C P P[V] P G G - - - S M[K] L D I G I I N E N Q[R] V S M S R - - - N I E S[R] S T S[P]	51
Hsvie_2	W[T] L H R N E D Q D R Y P S V I W E A K[C] R Y L[G] C V N A D G N V D Y H M N S V P I Q Q E I L V V R X G[H] Q P C[P] N[S] F	111	
Musctla8	W[T] L S R N E D P D R Y P S V I W E A Q C R H Q R C V N A E G[K] L D H H M N S V [Q] I Q Q E I L V V L K R E P E K C[P] F T[F]	111	
B18_F1	W[N] Y T V T W D P N R Y P S E[V] V Q[A] Q C R N L G C I N A Q G K E D I S M N S V P I Q Q E I T L V V R X G[K] H Q G C S V S F	111	
Hsvie_2	R L E K M L V T V G C T C V T P I V H N[Y] D	133	
Musctla8	R[V] E K M L V[G] V G C T C V S S[i] V R H A S	133	
B18_F1	Q L E K V L V T V G C T C V T P V I H H V Q	135	

FIGURE 1

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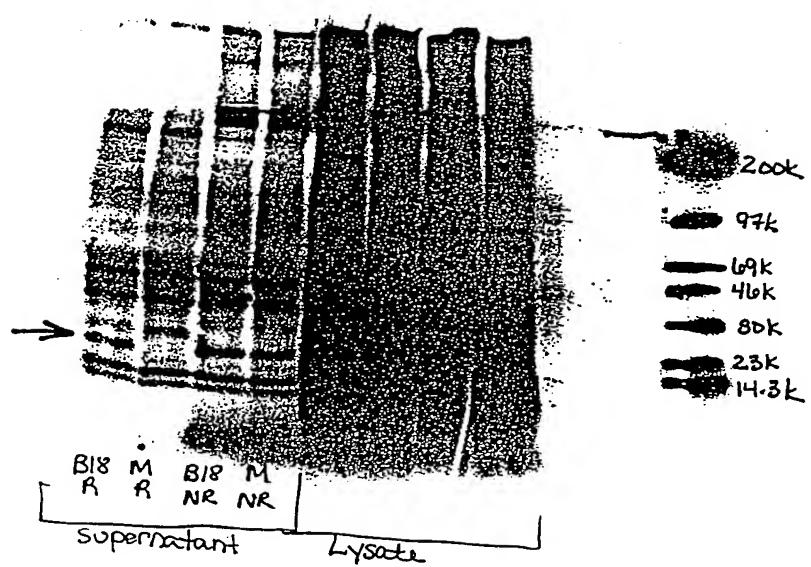


FIGURE 2

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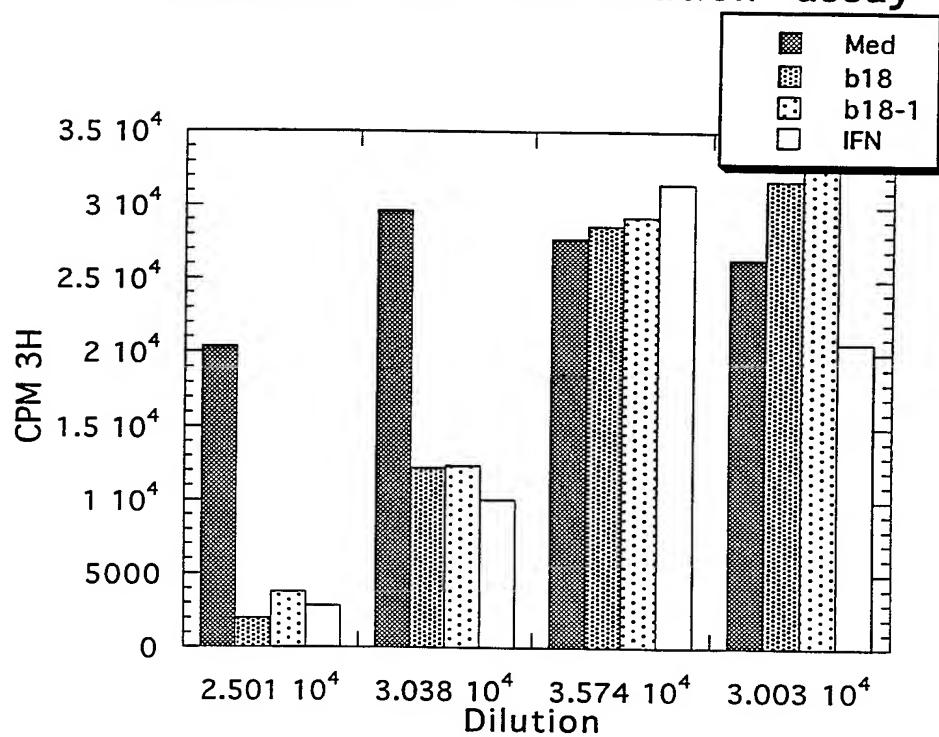
Endothelial Cell Proliferation assay

Fig. 3

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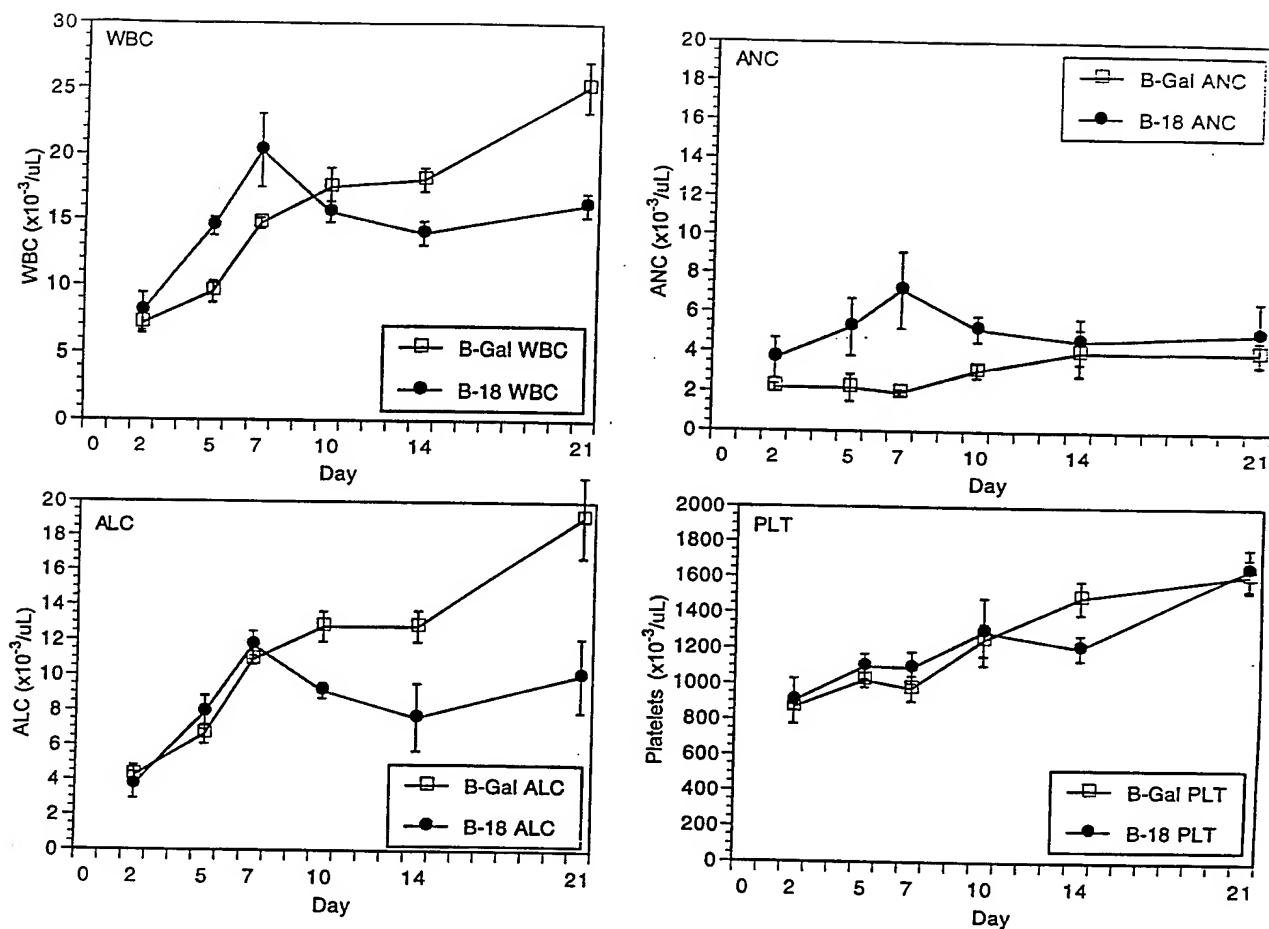


Fig. 4

TF-1 activity in CM from B18 induced HS-5 cells

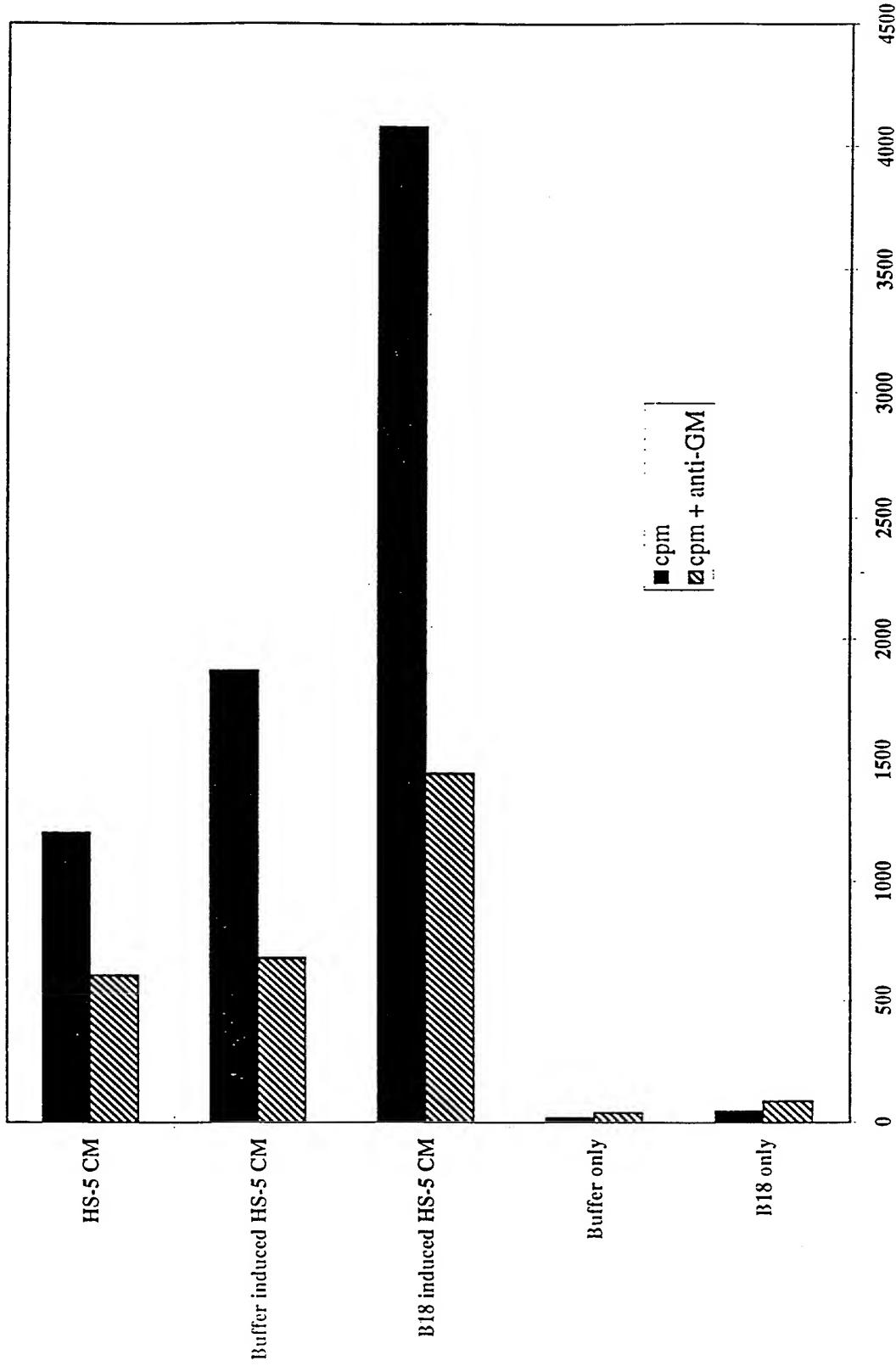


Fig. 5

IL6 Induction from B18 on MRC5 Cells - 24 & 48hrs

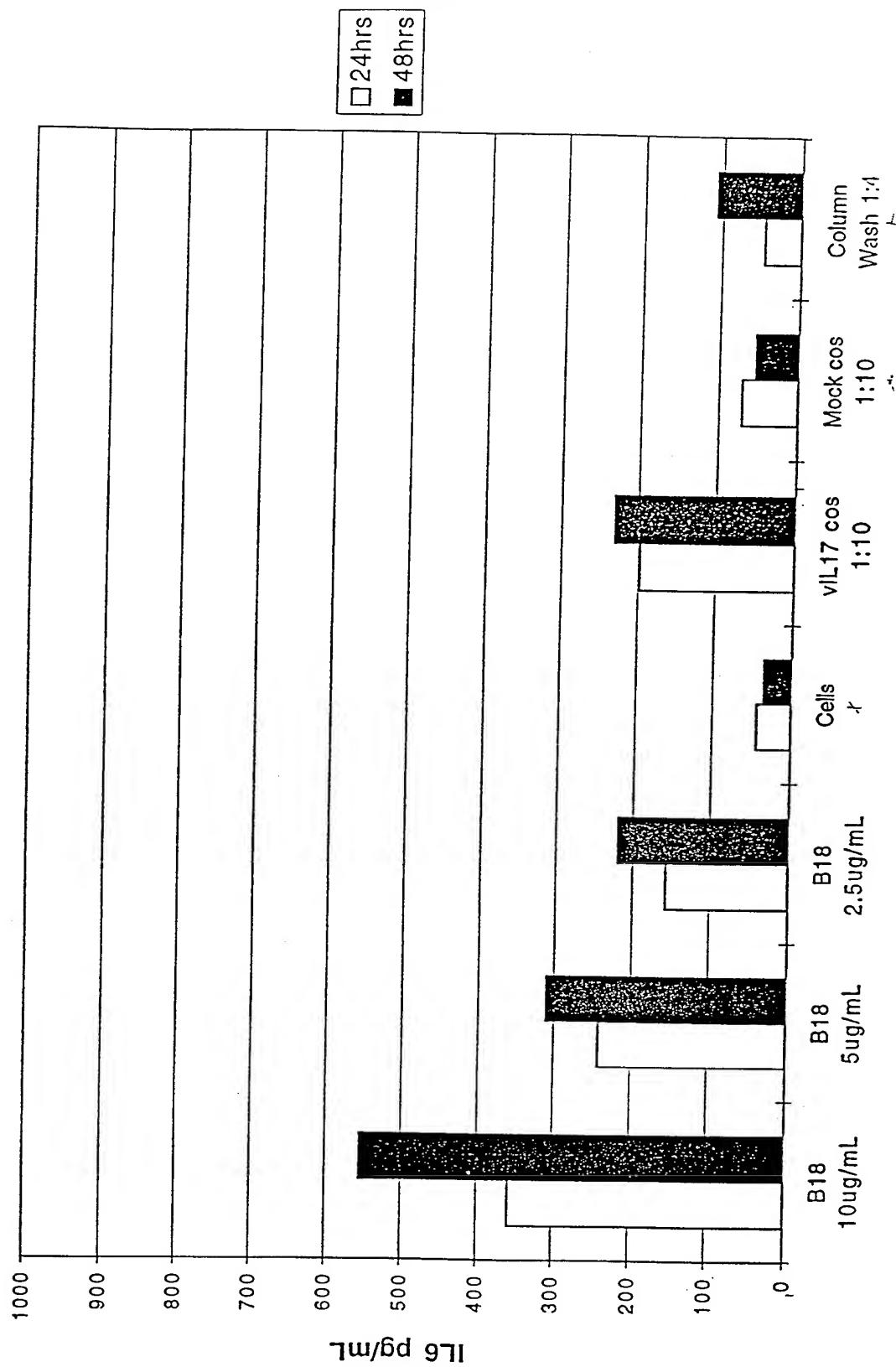


Fig. 6

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IL8 Production from B18 on MRC5 Cells - 24 & 48 hrs

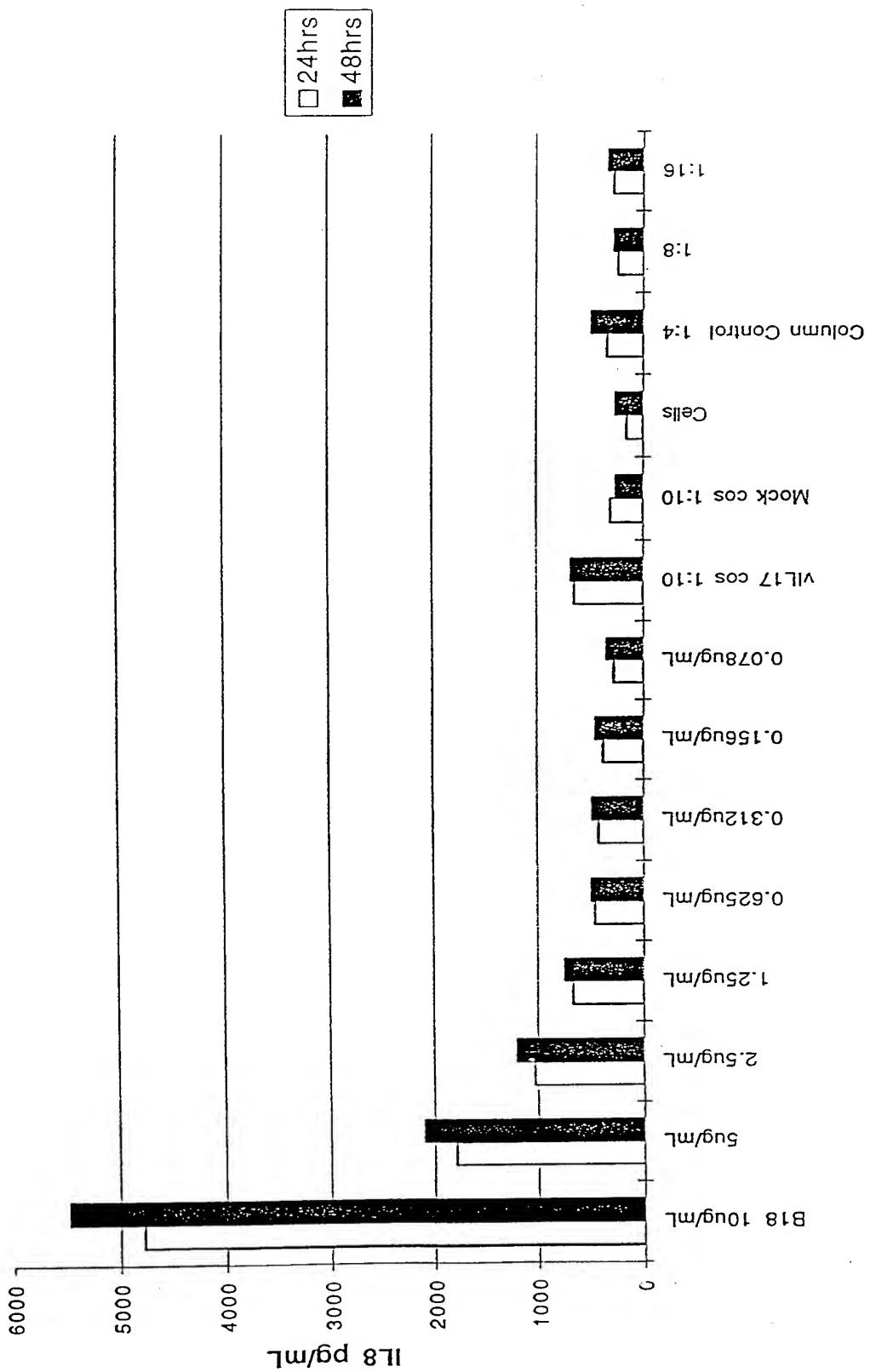


Fig. 7



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/19, 15/12, 15/24, C07K 14/52, 14/725, 14/54, A61K 38/17, 38/19, 38/20, C12N 5/10, C07K 16/24, 16/28, A61K 48/00		A3	(11) International Publication Number: WO 97/04097 (43) International Publication Date: 6 February 1997 (06.02.97)
(21) International Application Number: PCT/US96/11889 (22) International Filing Date: 18 July 1996 (18.07.96)		(81) Designated States: AU, CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 08/504,032 19 July 1995 (19.07.95) US 08/514,014 11 August 1995 (11.08.95) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 Cambridge Park Drive, Cambridge, MA 02140 (US).		(88) Date of publication of the international search report: 12 September 1997 (12.09.97)	
(72) Inventors: JACOBS, Kenneth; 151 Beaumont Avenue, Newton, MA 02160 (US). KELLEHER, Kerry; 50 Hurley Circle, Marlborough, MA 01752 (US). CARLIN, McKeough; 16 Chauncy Street #2, Cambridge, MA 02138 (US). GOLDMAN, Samuel; 9 Mohawk Drive, Acton, MA 01720 (US). PITTMAN, Debra; 20 N. Shore Road, Windham, NH 03087 (US). MI, Sha; 4 Vernon Road, Belmont, MA 02178 (US). NEBEN, Steven; 13 Duggan Road, Acton, MA 01720 (US). GIANNOTTI, JoAnn; 409 Arlington Street, Acton, MA 01720 (US). GOLDEN-FLEET, Margaret; 19 Usher Road, Medford, MA 02155 (US).			
(74) Agent: BROWN, Scott, A.; Genetics Institute, Inc., 87 Cambridge Park Drive, Cambridge, MA 02140 (US).			

(54) Title: HUMAN CTLA-8 AND USES OF CTLA-8-RELATED PROTEINS

(57) Abstract

Polynucleotides encoding human CTLA-8 and related proteins are disclosed. Human CTLA-8 proteins and methods for their production are also disclosed. Methods of treatment using human CTLA-8 proteins, rat CTLA-8 proteins and herpesvirus herpes CTLA-8 proteins are also provided.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/11889

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/19 C12N15/12 C12N15/24 C07K14/52 C07K14/725
C07K14/54 A61K38/17 A61K38/19 A61K38/20 C12N5/10
C07K16/24 C07K16/28 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 18826 A (SCHERING CORP ;INST NAT SANTE RECH MED (FR)) 13 July 1995 cited in the application	1-3,5-7, 12, 15-20, 23-25
A	see page 3, line 21 - page 4, line 11 see page 12, line 1 - page 16, line 6 see page 30, line 1-13 Seq.ID:2 see page 53 Seq.ID:4 see page 55 Seq.ID:5 see page 56 Seq.ID:7/8 see page 58 - page 59 ----	11,13,14 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- *E* earlier document but published on or after the international filing date
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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

1

Date of the actual completion of the international search

Date of mailing of the international search report

21 July 1997

25.07.97

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Authorized officer

Macchia, G

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/11889

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>THE JOURNAL OF IMMUNOLOGY, vol. 150, no. 12, 15 June 1993, pages 5445-5456, XP002035505 ROUVIER E. ET AL.: "CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a Herpesvirus Saimiri gene" cited in the application see page 5445 - page 5446, line 11 see page 5449, right-hand column, line 26 - page 5450 see page 5451; figure 3 see page 5453, left-hand column, line 10 - right-hand column, line 16 ---</p>	15-20
X	<p>JOURNAL OF VIROLOGY, vol. 66, no. 8, August 1992, pages 5047-5058, XP000615399 ALBRECHT J -C ET AL: "PRIMARY STRUCTURE OF THE HERPESVIRUS SAIMIRI GENOME"</p>	1
A	<p>Seq.ID:1 from nt.552 to nt.217 (reverse orientation) is 61.4% homologous to X64346 from nt.26931 to nt.27266. see page 5048, right-hand column, line 15-18 ORF13 see page 5049; table 1 ---</p>	18-20
E	<p>WO 97 07198 A (GENETICS INST) 27 February 1997 see page 8, line 15-31 see page 9, line 26 - page 11, line 7 see page 13, line 16-27 see page 18, line 10 - page 19, line 25 see page 25, line 11-15 see page 27, line 5-17 see page 31, line 27-34 Seq.ID:11-12 see page 49 - page 51 ---</p>	1-14,21, 22,24-27
P,X	<p>JOURNAL OF IMMUNOLOGY, vol. 155, no. 12, 15 December 1995, pages 5483-5486, XP000602481 YAO Z ET AL: "HUMAN IL-17: A NOVEL CYTOKINE DERIVED FROM T CELLS" see page 5483 see page 5484; figure 1 ---</p>	1-3,5-7, 23
	-/-	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/11889

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 183, no. 6, 1 June 1996, pages 2593-2603, XP002035506 FOSSIEZ F. ET AL.: "T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines" see abstract see page 2594; figure 1 ----	1-3,5-7, 15-20, 23,24
P,X	IMMUNITY, vol. 3, no. 6, 1 December 1995, pages 811-821, XP000579309 YAO Z ET AL: "HERPESVIRUS SAIMIRI ENCODES A NEW CYTOKINE, IL-17, WHICH BINDS TO A NOVEL CYTOKINE RECEPTOR" see page 811 see page 815, left-hand column, line 50 - page 818, left-hand column ----	15-20, 23,24
E	WO 96 29408 A (IMMUNEX CORP) 26 September 1996 see page 1, line 1 - page 2, line 33 Seq.ID:8 see page 36 ----	18-20
P,X	GENE, vol. 168, no. 2, 12 February 1996, pages 223-225, XP002035631 YAO ET AL.: "Complete nucleotide sequence of the mouse CTLA8 gene" see page 225; figure 2 -----	1,2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 11889

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 14-20, 23, 24
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 14-20, 2, 24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 11889

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

- 1) claims 1-14, 21, 22, 25-27 all totally; claim 24 partially.

Isolated polynucleotide comprising Seq.ID:1, homologue sequences and derivates. Vectors and transformed host cells. Process for producing human recombinant CTLA-8 protein. Isolated human CTLA-8 protein as in Seq.ID:2 and fragments. Pharmaceutical compositions and uses in therapy. Antibodies.

- 2) claims 15-17 all totally; claim 24 partially.

Therapeutical uses of the protein as in Seq.ID:4 or fragments.

- 3) claims 18-20 all totally; claim 24 partially.

Therapeutical uses of the protein as in Seq.ID:6 or fragments.

- 4) claims 23 all totally; claim 24 partially.

Therapeutical uses of IL-17 or active fragments.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/11889

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9518826 A	13-07-95	AU 1520895 A EP 0733069 A JP 9501572 T	01-08-95 25-09-96 18-02-97
WO 9707198 A	27-02-97	AU 6712396 A AU 6768596 A WO 9704097 A	18-02-97 12-03-97 06-02-97
WO 9629408 A	26-09-96	AU 5526396 A	08-10-96



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: HUMAN CTLA-8 AND USES OF CTLA-8-RELATED PROTEINS

(57) Abstract

Polynucleotides encoding human CTLA-8 and related proteins are disclosed. Human CTLA-8 proteins and methods for their production are also disclosed. Methods of treatment using human CTLA-8 proteins, rat CTLA-8 proteins and herpesvirus herpes CTLA-8 proteins are also provided.

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HUMAN CTLA-8 AND USES OF CTLA-8-RELATED PROTEINS

This application is a continuation-in-part of application Ser. No. 08/504,032, filed July 19, 1995, and a continuation-in-part of application Ser. No. 08/514,014, filed August 11, 1995.

Field of the Invention

5 The present invention relates to human CTLA-8 proteins, nucleic acids encoding such proteins, methods of treatment using such proteins. The invention also relates to the use of rat CTLA-8 proteins and herpesvirus *Saimiri* ORF13 proteins in methods of treatment.

10 Background of the Invention

Cytokines are secreted proteins which act on specific hematopoietic target cells to cause a differentiation event or on other target cells to induce a particular physiological response, such as secretion of proteins characteristic of inflammation. Cytokines, also variously known as lymphokines, hematopoietins, interleukins, colony 15 stimulating factors, and the like, can be important therapeutic agents, especially for diseases or conditions in which a specific cell population is depleted. For example, erythropoietin, G-CSF, and GM-CSF, have all become important for treatment of anemia and leukopenia, respectively. Other cytokines such as interleukin-3, interleukin-6, interleukin-11 and interleukin-12 show promise in treatment of 20 conditions such as thrombocytopenia and modulation of immune response.

For these reasons a significant research effort has been expended in searching for novel cytokines and cloning the DNAs which encode them. In the past, novel cytokines were identified by assaying a particular cell such as a bone marrow cell, for a measurable response, such as proliferation. The search for novel cytokines has thus 5 been limited by the assays available, and if a novel cytokine has an activity which is unmeasurable by a known assay, the cytokine remains undetectable. In a newer approach, cDNAs encoding cytokines have been detected using the polymerase chain

reaction (PCR) and oligonucleotide primers having homology to shared motifs of known cytokines or their receptors. The PCR approach is also limited by the necessity for knowledge of previously cloned cytokines in the same protein family. Cytokines have also been cloned using subtractive hybridization to construct and screen cDNA libraries, or they can potentially be cloned using PCR followed by gel electrophoresis to detect differentially expressed genes. The subtractive hybridization methods are based on the assumption that cytokine mRNAs are those that are differentially expressed, and these methods do not require any prior knowledge of the sequence of interest. However, many cytokines may be encoded by mRNAs which are not differentially expressed, and thus are undetectable using these methods.

It would be desirable to develop new methods for identifying novel cytokines and other secreted factors and to isolate polynucleotides encoding them.

Summary of the Invention

In developing the present invention, methods were employed which selectively identify polynucleotides which encode secreted proteins. One such polynucleotide was isolated which encodes "human CTLA-8." In accordance with the present invention, polynucleotides encoding human CTLA-8 and active fragments thereof are disclosed. "CTLA-8" is used throughout the present specification to refer to both proteins and polynucleotides encoding those proteins and to refer to proteins and polynucleotides from all mammalian species.

In certain embodiments, the present invention provides an isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

- (a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 146 to nucleotide 544;
- (b) a nucleotide sequence capable of hybridizing to a nucleic acid sequence specified in (a);
- (c) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code; and
- (d) an allelic variant of the nucleotide sequence specified in (a).

Preferably, the polynucleotide of the invention encodes a protein having CTLA-8 activity. In other embodiments the polynucleotide is operably linked to an expression control sequence. In other preferred embodiments, the polynucleotide is contained in a vector suitable for *in vivo* expression in a mammalian subject. Polynucleotides comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 55 to nucleotide 544, the nucleotide sequence of SEQ ID NO:1 from nucleotide 139 to nucleotide 544 or the nucleotide sequence of SEQ ID NO:1 from nucleotide 86 to nucleotide 544 are particularly preferred.

Host cells transformed with the polynucleotides of the invention are also provided, including mammalian cells.

Processes are also provided for producing a human CTLA-8 protein, said processes comprising:

- (a) growing a culture of the host cell of the invention in a suitable culture medium; and
- 15 (b) purifying the human CTLA-8 protein from the culture.

Isolated human CTLA-8 protein is also provided which comprising an amino acid sequence selected from the group consisting of:

- (a) the amino acid sequence of SEQ ID NO:2;
- (b) the amino acid sequence of SEQ ID NO:2 from amino acids 11 to 163;
- 20 (c) the amino acid sequence of SEQ ID NO:2 from amino acids 29 to 163;
- (d) the amino acid sequence of SEQ ID NO:2 from amino acids 31 to 163;
and
- (e) fragments of (a), (b), (c) or (d) having CTLA-8 activity.

25 Proteins comprising the amino acid sequence of SEQ ID NO:2 and comprising the sequence from amino acids 29 to 163, from amino acid 31 to 163, or from amino acids 11 to 163 of SEQ ID NO:2 are particularly preferred. Preferably, the protein has CTLA-8 activity. Pharmaceuticals composition comprising a human CTLA-8 protein of the invention and a pharmaceutically acceptable carrier are also provided.

30 Compositions are also disclosed which comprise an antibody which specifically reacts with a human CTLA-8 protein of the invention.

Methods of treating a mammalian subject are also provided which comprise administering a therapeutically effective amount of a pharmaceutical composition comprising a human CTLA-8 protein.

Rat CTLA-8 and active (*i.e.*, having CTLA-8 activity) fragments thereof may 5 also be used in such methods of treatment. Preferably the rat protein is administered as a composition comprising a pharmaceutically acceptable carrier and a protein comprising an amino acid sequence selected from the group consisting of:

10 (a) the amino acid sequence of SEQ ID NO:4;
(b) the amino acid sequence of SEQ ID NO:4 from amino acids 18 to 150;

and

(c) fragments of (a) or (b) having CTLA-8 activity.

Herpesvirus *Saimiri* ORF13, referred to herein as "herpes CTLA-8", and active (i.e., having CTLA-8 activity) fragments thereof and active fragments thereof may also be used in such methods of treatment. Preferably the herpes CTLA-8 protein is 15 administered as a composition comprising a pharmaceutically acceptable carrier and a protein comprising an amino acid sequence selected from the group consisting of:

15 (a) the amino acid sequence of SEQ ID NO:6;
(b) the amino acid sequence of SEQ ID NO:6 from amino acids 19 to 151;
and
20 (c) fragments of (a) or (b) having CTLA-8 activity.

The invention also provides a method of treating a mammalian subject comprising administering a therapeutically effective amount of a composition comprising a pharmaceutically acceptable carrier and IL-17 or an active fragment thereof.

25 In methods of treatment provided by the present invention, preferably the subject is treated to produce an effect selected from the group consisting of inhibition of angiogenesis, inhibition of growth or proliferation of vascular endothelial cells, inhibition of tumor growth, inhibition of angiogenesis-dependent tissue growth, proliferation of myeloid cells or progenitors, proliferation of erythroid cells or 30 progenitors, proliferation of lymphoid cells or progenitors, induction of IFN γ production, induction of IL-3 production and induction of GM-CSF production.

Brief Description of the Figures

Fig. 1 is a comparison of homologous regions of the amino acid sequences of human CTLA-8 (indicated as "B18_F1"), rat CTLA-8 (indicated as "Musctla8") and herpes CTLA-8 (indicated as "Hsvie_2").

5 Fig. 2 depicts autoradiographs demonstrating expression of human CTLA-8 in COS cells.

Fig. 3 presents data relating to the ability of human CTLA-8 to inhibit angiogenesis.

10 Figs. 4 and 5 present data relating to the ability of human CTLA-8 to produce or induce hematopoietic activity.

Figs. 6 and 7 present data demonstrating the ability of human CTLA-8 to induce production of IL-6 and IL-8.

Detailed Description of Preferred Embodiments

15 The inventors of the present application have identified and provided a polynucleotide encoding a human CTLA-8 protein. SEQ ID NO:1 provides the nucleotide sequence of a cDNA encoding the human CTLA-8 protein. SEQ ID NO:2 provides the amino acid sequence of the human CTLA-8 protein. Alternatively, the initiating methionine may be at amino acid 11 of SEQ ID NO:2. On the basis of 20 amino terminal sequencing, the mature protein sequence is believed to begin at amino acid 31 of SEQ ID NO:2 (encoded by the sequence beginning with nucleotide 146 of SEQ ID NO:1).

The region from amino acid 29 to amino acid 163 of human CTLA-8 (SEQ ID NO:2) shows marked homology to portions of rat CTLA-8 (amino acids 18 to 150 of 25 SEQ ID NO:4) and herpesvirus *Saimiri* ORF13 ("herpes CTLA-8") (amino acids 19 to 151 of SEQ ID NO:5). A cDNA sequence encoding rat CTLA-8 is listed at SEQ ID NO:3 and its corresponding amino acid sequence is reported at SEQ ID NO:4. A cDNA sequence encoding herpes CTLA-8 is listed at SEQ ID NO:5 and its corresponding amino acid sequence is reported at SEQ ID NO:6. Homology between 30 rat CTLA-8 and herpes CTLA-8 was reported by Rouvier et al., J. Immunol. 1993, 150, 5445-5456.

Applicants had previously incorrectly identified the rat sequences of SEQ ID NO:3 and SEQ ID NO:4 as applying to murine CTLA-8. Applicants' human CTLA-8 (B18) does also show homology to the true murine CTLA-8 sequence.

Golstein et al. (WO95/18826; Fossiez et al., Microbial Evasion and Subversion of Immunity 544:3222 (Abstract)) have also reported a species they initially identified as "human CTLA-8." However, examination of the sequence of the Golstein et al. species and the human CTLA-8 (B18) sequence of the present invention readily reveals that they are two different proteins, although they are homologous with each other and with the rat CTLA-8 and herpes CTLA-8 identified herein. The Golstein et al. species has now been renamed as interleukin-17 (IL-17). Because of the homology between applicants' human CTLA-8 (B18) and IL-17, these proteins are expected to share some activities.

It has also been preliminarily determined that human CTLA-8 (B18) forms homodimers when expressed. As a result, human CTLA-8 proteins may possess activity in either monomeric or dimeric forms. Human CTLA-8 proteins can also be produced as heterodimers with rat and herpes CTLA-8 proteins and with human IL-17. These heterodimers are also expected to have activities of the proteins of which they are comprised.

Forms of human CTLA-8 protein of less than full length are encompassed within the present invention and may be produced by expressing a corresponding fragment of the polynucleotide encoding the human CTLA-8 protein (SEQ ID NO:1). These corresponding polynucleotide fragments are also part of the present invention. Modified polynucleotides as described above may be made by standard molecular biology techniques, including site-directed mutagenesis methods which are known in the art or by the polymerase chain reaction using appropriate oligonucleotide primers.

For the purposes of the present invention, a protein has "CTLA-8 activity" if it either (1) displays biological activity in a factor-dependent cell proliferation assay (preferably an assay in which full-length the corresponding species full-length CTLA-8 is active) (including without limitation those assays described below), or (2) induces expression or secretion of γ -IFN, or (3) displays chemoattractant of chemotactic activity in a chemoattraction or chemotaxis assay (preferably as assay in which full-

length the corresponding species full-length CTLA-8 is active) or (4) induces expression of secretion of IL-3 or GM-CSF.

Human CTLA-8 protein or fragments thereof having CTLA-8 activity may be fused to carrier molecules such as immunoglobulins. For example, human CTLA-8 protein may be fused through "linker" sequences to the Fc portion of an immunoglobulin.

The invention also encompasses allelic variations of the nucleotide sequence as set forth in SEQ ID NO:1, that is, naturally-occurring alternative forms of the isolated polynucleotide of SEQ ID NO:1 which also encode human CTLA-8 or CTLA-8 proteins having CTLA-8 activity. Also included in the invention are isolated polynucleotides which hybridize to the nucleotide sequence set forth in SEQ ID NO:1 under highly stringent (0.2xSSC at 65°C), stringent (e.g. 4xSSC at 65°C or 50% formamide and 4xSSC at 42°C), or relaxed (4xSSC at 50°C or 30-40% formamide and 4xSSC at 42°C) conditions. Isolated polynucleotides which encode human CTLA-8 protein but which differ from the nucleotide sequence set forth in SEQ ID NO:1 by virtue of the degeneracy of the genetic code are also encompassed by the present invention. Variations in the nucleotide sequence as set forth in SEQ ID NO:1 which are caused by point mutations or by induced modifications which enhance CTLA-8 activity, half-life or production level are also included in the invention.

The isolated polynucleotides of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the CTLA-8 protein recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means enzymatically or chemically ligated to form a covalent bond between the isolated polynucleotide of the invention and the expression control sequence, in such a way that the CTLA-8 protein is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the human CTLA-8 protein. Any cell type capable of expressing functional human CTLA-8 protein may be used. Suitable mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, 5 human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK, Rat2, BaF3, 32D, FDCP-1, PC12 or C2C12 cells.

The human CTLA-8 protein may also be produced by operably linking the 10 isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, California, U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas 15 Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference. Soluble forms of the human CTLA-8 protein may also be produced in insect cells using appropriate isolated polynucleotides as described above.

Alternatively, the human CTLA-8 protein may be produced in lower 20 eukaryotes such as yeast or in prokaryotes such as bacteria. Suitable yeast strains include *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces* strains, *Candida*, or any yeast strain capable of expressing heterologous proteins. Suitable bacterial strains include *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, or any bacterial strain capable of expressing heterologous proteins.

The human CTLA-8 protein of the invention may also be expressed as a 25 product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide sequence encoding the human CTLA-8 protein.

The human CTLA-8 protein of the invention may be prepared by growing a 30 culture of transformed host cells under culture conditions necessary to express the desired protein. The resulting expressed protein may then be purified from the culture medium or cell extracts. Soluble forms of the human CTLA-8 protein of the invention

can be purified from conditioned media. Membrane-bound forms of human CTLA-8 protein of the invention can be purified by preparing a total membrane fraction from the expressing cell and extracting the membranes with a non-ionic detergent such as Triton X-100.

5 The human CTLA-8 protein can be purified using methods known to those skilled in the art. For example, the human CTLA-8 protein of the invention can be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium.
10 Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or
15 carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the human CTLA-8 protein from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether,
20 or propyl ether; or by immunoaffinity chromatography. Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the human CTLA-8 protein. Some or all of the foregoing purification steps, in various combinations or with other known
25 methods, can also be employed to provide a substantially purified isolated recombinant protein.

Preferably, the human CTLA-8 protein is purified so that it is substantially free of other mammalian proteins.

It is believed that human CTLA-8, active fragments and variants thereof, and
30 CTLA-8 related proteins (such as, for example, rat CTLA-8 and herpes CTLA-8) (collectively "CTLA-8 proteins") possess or induce cytokine activities. Human

5 CTLA-8 expression correlated with γ -IFN expression in induced primary cells and can induce the expression of IL-3 and/or GM-CSF, which expression can in turn produce effects associated with the induced cytokine. Therefore, human CTLA-8 and CTLA-8 related proteins may have an effect on proliferation or function of myeloid cells, erythroid cells, lymphoid cells and their progenitors. Human CTLA-8 proteins may also play a role in formation of platelets or their progenitors.

10 A protein of the present invention may exhibit cytokine, cell proliferation (either inducing or inhibiting) or cell differentiation (either inducing or inhibiting) activity or may induce production of other cytokines in certain cell populations. Many protein factors discovered to date, including all known cytokines, have exhibited activity in one or more factor dependent cell proliferation assays, and hence the assays serve as a convenient confirmation of cytokine activity. The activity of a protein of the present invention is evidenced by any one of a number of routine factor dependent cell proliferation assays for cell lines including, without limitation, 32D, DA2, DA1G, 15 T10, B9, B9/11, BaF3, MC9/G, M+ (preB M+), 2E8, RB5, DA1, 123, T1165, HT2, CTLL2, TF-1, Mo7e and CMK.

The activity of a protein of the invention may, among other means, be measured by the following methods:

20 Assays for T-cell or thymocyte proliferation include without limitation those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Takai et al., J. Immunol. 137:3494-3500, 1986; Bertagnolli et al., J. Immunol. 145:1706-1712, 1990; 25 Bertagnolli et al., Cellular Immunology 133:327-341, 1991; Bertagnolli, et al., J. Immunol. 149:3778-3783, 1992; Bowman et al., J. Immunol. 152: 1756-1761, 1994.

Assays for cytokine production and/or proliferation of spleen cells, lymph node cells or thymocytes include, without limitation, those described in: Polyclonal T cell stimulation, Kruisbeek, A.M. and Shevach, E.M. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 3.12.1-3.12.14, John Wiley and Sons, Toronto. 1994; and Measurement of mouse and human Interferon γ , Schreiber, R.D.

In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.8.1-6.8.8, John Wiley and Sons, Toronto. 1994.

Assays for proliferation and differentiation of hematopoietic and lymphopoietic cells include, without limitation, those described in: Measurement of Human and Murine Interleukin 2 and Interleukin 4, Bottomly, K., Davis, L.S. and Lipsky, P.E. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.3.1-6.3.12, John Wiley and Sons, Toronto. 1991; deVries et al., *J. Exp. Med.* 173:1205-1211, 1991; Moreau et al., *Nature* 336:690-692, 1988; Greenberger et al., *Proc. Natl. Acad. Sci. U.S.A.* 80:2931-2938, 1983; Measurement of mouse and human interleukin 6 - Nordan, R. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.6.1-6.6.5, John Wiley and Sons, Toronto. 1991; Smith et al., *Proc. Natl. Acad. Sci. U.S.A.* 83:1857-1861, 1986; Measurement of human Interleukin 11 - Bennett, F., Giannotti, J., Clark, S.C. and Turner, K. J. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.15.1 John Wiley and Sons, Toronto. 1991; Measurement of mouse and human Interleukin 9 - Ciarletta, A., Giannotti, J., Clark, S.C. and Turner, K.J. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 6.13.1, John Wiley and Sons, Toronto. 1991.

Assays for T-cell clone responses to antigens (which will identify, among others, proteins that affect APC-T cell interactions as well as direct T-cell effects by measuring proliferation and cytokine production) include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober

Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function; Chapter 6, Cytokines and their cellular receptors; Chapter 7, Immunologic studies in Humans); Weinberger et al., *Proc. Natl. Acad. Sci. USA* 77:6091-6095, 1980; Weinberger et al., *Eur. J. Immun.* 11:405-411, 1981; Takai et al., *J. Immunol.* 137:3494-3500, 1986; Takai et al., *J. Immunol.* 140:508-512, 1988.

A protein of the present invention may also exhibit immune stimulating or immune suppressing activity, including without limitation the activities for which assays are described herein. A protein may be useful in the treatment of various

immune deficiencies and disorders (including severe combined immunodeficiency (SCID)), e.g., in regulating (up or down) growth and proliferation of T and/or B lymphocytes, as well as effecting the cytolytic activity of NK cells and other cell populations. These immune deficiencies may be genetic or be caused by viral (e.g., 5 HIV) as well as bacterial or fungal infections, or may result from autoimmune disorders. More specifically, infectious diseases causes by viral, bacterial, fungal or other infection may be treatable using a protein of the present invention, including infections by HIV, hepatitis viruses, herpes viruses, mycobacteria, leshmania, malaria and various fungal infections such as candida. Of course, in this regard, a protein of 10 the present invention may also be useful where a boost to the immune system generally would be indicated, i.e., in the treatment of cancer.

Autoimmune disorders which may be treated using a protein of the present invention include, for example, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, autoimmune pulmonary inflammation, Guillain-Barre syndrome, 15 autoimmune thyroiditis, insulin dependent diabetes mellitis, myasthenia gravis, graft-versus-host disease and autoimmune inflammatory eye disease. Such a protein of the present invention may also be useful in the treatment of allergic reactions and conditions, such as asthma or other respiratory problems. Other conditions, in which immune suppression is desired (including, for example, asthma and related respiratory 20 conditions), may also be treatable using a protein of the present invention.

A protein of the present invention may also suppress chronic or acute inflammation, such as, for example, that associated with infection (such as septic shock or systemic inflammatory response syndrome (SIRS)), inflammatory bowel disease, Crohn's disease or resulting from over production of cytokines such as TNF 25 or IL-1 (such as the effect demonstrated by IL-11).

The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for thymocyte or splenocyte cytotoxicity include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, 30 A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte

Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Herrmann et al., Proc. Natl. Acad. Sci. USA 78:2488-2492, 1981; Herrmann et al., J. Immunol. 128:1968-1974, 1982; Handa et al., J. Immunol. 135:1564-1572, 1985; Takai et al., J. Immunol. 137:3494-3500, 1986; Takai et al., J. Immunol. 140:508-512, 1988;

5 Herrmann et al., Proc. Natl. Acad. Sci. USA 78:2488-2492, 1981; Herrmann et al., J. Immunol. 128:1968-1974, 1982; Handa et al., J. Immunol. 135:1564-1572, 1985; Takai et al., J. Immunol. 137:3494-3500, 1986; Bowman et al., J. Virology 61:1992-1998; Takai et al., J. Immunol. 140:508-512, 1988; Bertagnolli et al., Cellular Immunology 133:327-341, 1991; Brown et al., J. Immunol. 153:3079-3092, 1994.

10 Assays for T-cell-dependent immunoglobulin responses and isotype switching (which will identify, among others, proteins that modulate T-cell dependent antibody responses and that affect Th1/Th2 profiles) include, without limitation, those described in: Maliszewski, J. Immunol. 144:3028-3033, 1990; and Assays for B cell function: *In vitro* antibody production, Mond, J.J. and Brunswick, M. In *Current Protocols in Immunology*. J.E.e.a. Coligan eds. Vol 1 pp. 3.8.1-3.8.16, John Wiley and Sons, Toronto. 1994.

15 Mixed lymphocyte reaction (MLR) assays (which will identify, among others, proteins that generate predominantly Th1 and CTL responses) include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Takai et al., J. Immunol. 137:3494-3500, 1986; Takai et al., J. Immunol. 140:508-512, 1988; Bertagnolli et al., J. Immunol. 149:3778-3783, 1992.

20 Dendritic cell-dependent assays (which will identify, among others, proteins expressed by dendritic cells that activate naive T-cells) include, without limitation, those described in: Guery et al., J. Immunol. 134:536-544, 1995; Inaba et al., Journal of Experimental Medicine 173:549-559, 1991; Macatonia et al., Journal of Immunology 154:5071-5079, 1995; Porgador et al., Journal of Experimental Medicine 182:255-260, 1995; Nair et al., Journal of Virology 67:4062-4069, 1993; Huang et al., Science 264:961-965, 1994; Macatonia et al., Journal of Experimental Medicine

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169:1255-1264, 1989; Bhardwaj et al., Journal of Clinical Investigation 94:797-807, 1994; and Inaba et al., Journal of Experimental Medicine 172:631-640, 1990.

Assays for lymphocyte survival/apoptosis (which will identify, among others, proteins that prevent apoptosis after superantigen induction and proteins that regulate lymphocyte homeostasis) include, without limitation, those described in: Darzynkiewicz et al., Cytometry 13:795-808, 1992; Gorczyca et al., Leukemia 7:659-670, 1993; Gorczyca et al., Cancer Research 53:1945-1951, 1993; Itoh et al., Cell 66:233-243, 1991; Zacharchuk, Journal of Immunology 145:4037-4045, 1990; Zamai et al., Cytometry 14:891-897, 1993; Gorczyca et al., International Journal of Oncology 1:639-648, 1992.

Assays for proteins that influence early steps of T-cell commitment and development include, without limitation, those described in: Antica et al., Blood 84:111-117, 1994; Fine et al., Cellular Immunology 155:111-122, 1994; Galy et al., Blood 85:2770-2778, 1995; Toki et al., Proc. Nat. Acad. Sci. USA 88:7548-7551, 1991.

A protein of the present invention may be useful in regulation of hematopoiesis and, consequently, in the treatment of myeloid or lymphoid cell deficiencies. Even marginal biological activity in support of colony forming cells or of factor-dependent cell lines indicates involvement in regulating hematopoiesis, e.g. in supporting the growth and proliferation of erythroid progenitor cells alone or in combination with other cytokines, thereby indicating utility, for example, in treating various anemias or for use in conjunction with irradiation/chemotherapy to stimulate the production of erythroid precursors and/or erythroid cells; in supporting the growth and proliferation of myeloid cells such as granulocytes and monocytes/macrophages (i.e., traditional CSF activity) useful, for example, in conjunction with chemotherapy to prevent or treat consequent myelo-suppression; in supporting the growth and proliferation of megakaryocytes and consequently of platelets thereby allowing prevention or treatment of various platelet disorders such as thrombocytopenia, and generally for use in place of or complimentarily to platelet transfusions; and/or in supporting the growth and proliferation of hematopoietic stem cells which are capable of maturing to any and all of the above-mentioned hematopoietic cells and therefore find therapeutic utility

in various stem cell disorders (such as those usually treated with transplantation, including, without limitation, aplastic anemia and paroxysmal nocturnal hemoglobinuria), as well as in repopulating the stem cell compartment post irradiation/chemotherapy, either *in-vivo* or *ex-vivo* (i.e. in conjunction with bone marrow transplantation) as normal cells or genetically manipulated for gene therapy.

5 The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for proliferation and differentiation of various hematopoietic lines are cited above.

10 Assays for embryonic stem cell differentiation (which will identify, among others, proteins that influence embryonic differentiation hematopoiesis) include, without limitation, those described in: Johansson et al. *Cellular Biology* 15:141-151, 1995; Keller et al., *Molecular and Cellular Biology* 13:473-486, 1993; McClanahan et al., *Blood* 81:2903-2915, 1993.

15 Assays for stem cell survival and differentiation (which will identify, among others, proteins that regulate lympho-hematopoiesis) include, without limitation, those described in: Methylcellulose colony forming assays, Freshney, M.G. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 265-268, Wiley-Liss, Inc., New York, NY. 1994; Hirayama et al., *Proc. Natl. Acad. Sci. USA* 89:5907-5911, 1992; Primitive hematopoietic colony forming cells with high proliferative potential, McNiece, I.K. and Briddell, R.A. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 23-39, Wiley-Liss, Inc., New York, NY. 1994; Neben et al., *Experimental Hematology* 22:353-359, 1994; Cobblestone area forming cell assay, Ploemacher, R.E. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 1-21, Wiley-Liss, Inc., New York, NY. 1994; Long term bone marrow cultures in the presence of stromal cells, Spooncer, E., Dexter, M. and Allen, T. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 163-179, Wiley-Liss, Inc., New York, NY. 1994; Long term culture initiating cell assay, Sutherland, H.J. In *Culture of Hematopoietic Cells*. R.I. Freshney, et al. eds. Vol pp. 139-162, Wiley-Liss, Inc., New York, NY. 1994.

CTLA-8 proteins are useful in the treatment of various immune deficiencies and disorders (including SCID), *e.g.*, in regulating (up or down) growth, proliferation and/or activity of T and/or B lymphocytes, as well as the cytolytic activity of NK cells. These immune deficiencies may be caused by viral (*e.g.*, HIV) as well as bacterial infections, or may result from autoimmune disorders. More specifically, infectious diseases caused by viral, bacterial, fungal or other infection may be treatable using CTLA-8 proteins, including infections by HIV, hepatitis, influenza, CMV, herpes, mycobacterium, leishmaniasis, malaria and various fungal infections (such as candida). Of course, in this regard, the CTLA-8 proteins may also be useful where a boost to the immune system generally would be indicated, *i.e.*, in the treatment of cancer or as an adjuvant to vaccines. Autoimmune disorders which may be treated using factors of the present invention include, for example, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, autoimmune pulmonary inflammation, Guillain-Barre syndrome, autoimmune thyroiditis, insulin dependent diabetes melitis and autoimmune inflammatory eye disease. The CTLA-8 proteins are also expected to be useful in the treatment of allergic reactions and conditions.

CTLA-8 proteins are also expected to have chemotactic activity. A protein or peptide has "chemotactic activity," as used herein, if it can stimulate, directly or indirectly, the directed orientation or movement of cells, including myeloid and lymphoid cells. Preferably, the protein or peptide has the ability to directly stimulate directed movement of cells (particularly T-cells). Whether a particular protein or peptide has chemotactic activity for cells can be readily determined by employing such protein or peptide in any known assay for cell chemotaxis.

CTLA-8 proteins also inhibit growth and proliferation of vascular endothelial cells. As a result, human CTLA-8 proteins are effective in inhibiting angiogenesis (*i.e.*, vascular formation). This activity will also be useful in the treatment of tumors and other conditions in which angiogenesis is involved. Inhibition of angiogenesis by human CTLA-8 proteins will also result in inhibition or prevention of the condition to which normal angiogenesis would contribute.

Isolated CTLA-8 proteins, purified from cells or recombinantly produced, may be used as a pharmaceutical composition when combined with a pharmaceutically

acceptable carrier. Such a composition may contain, in addition to CTLA-8 protein and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration. The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, G-CSF, γ -IFN, stem cell factor, and erythropoietin. The pharmaceutical composition may contain thrombolytic or anti-thrombotic factors such as plasminogen activator and Factor VIII. The pharmaceutical composition may further contain other anti-inflammatory agents. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with CTLA-8 protein, or to minimize side effects caused by the CTLA-8 protein. Conversely, CTLA-8 protein may be included in formulations of the particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which CTLA-8 protein is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, e.g., amelioration of symptoms of,

healing of, or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of CTLA-8 protein is administered to a mammal. CTLA-8 protein may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines, other hematopoietic factors or vaccine components (such as antigens or other adjuvants), CTLA-8 protein may be administered either simultaneously with the cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering CTLA-8 protein in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

Administration of CTLA-8 protein used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of CTLA-8 protein is administered orally, CTLA-8 protein will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% CTLA-8 protein, and preferably from about 25 to 90% CTLA-8 protein. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol,

propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of CTLA-8 protein, and preferably from about 1 to 50% CTLA-8 protein.

When a therapeutically effective amount of CTLA-8 protein is administered
5 by intravenous, cutaneous or subcutaneous injection, CTLA-8 protein will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in
10 addition to CTLA-8 protein an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

15 The amount of CTLA-8 protein in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of CTLA-8 protein with which to treat each individual patient. Initially, the attending physician will
20 administer low doses of CTLA-8 protein and observe the patient's response. Larger doses of CTLA-8 protein may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not generally increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.1 µg to about 100 mg of
25 CTLA-8 protein per kg body weight, preferably about 0.1 µg to about 10 mg of CTLA-8 protein per kg body weight, more preferably about 0.1 µg to about 100 µg of CTLA-8 protein per kg body weight, most preferably preferably about 0.1 µg to about 10 µg of CTLA-8 protein per kg body weight.

30 The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It

is contemplated that the duration of each application of the CTLA-8 protein will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

5 CTLA-8 protein of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the CTLA-8 protein and which may inhibit CTLA-8 binding to its receptor. Such antibodies are also useful for performing diagnostics assays for CTLA-8 in accordance with known methods. Such antibodies may be obtained using the entire CTLA-8 protein as an 10 immunogen, or by using fragments of human CTLA-8 protein. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Additional peptide immunogens may be generated by replacing tyrosine residues with sulfated tyrosine residues. Methods for synthesizing such peptides are known in the art, for 15 example, as in R.P. Merrifield, J.Amer.Chem.Soc. 85, 2149-2154 (1963); J.L. Krstenansky, et al., FEBS Lett. 211, 10 (1987).

Neutralizing or non-neutralizing antibodies (preferably monoclonal antibodies) binding to human CTLA-8 protein may also be useful therapeutics for certain tumors and also in the treatment of conditions described above. These neutralizing 20 monoclonal antibodies are capable of blocking the ligand binding to the human CTLA-8 protein or may promote clearance of protein from the patient.

Because of their homology to human CTLA-8, rat CTLA-8 proteins, herpes 25 CTLA-8 proteins and IL-17 proteins (the "human CTLA-8" of Golstein et al., supra) will also possess CTLA-8 activity as described above. As a result, rat and herpes CTLA-8 proteins and IL-17 proteins, as well as active fragments and variants thereof, can be used in preparation of pharmaceutical compositions and in methods of treatment as described for human CTLA-8. Rat and herpes CTLA-8 proteins, and active fragments and variants thereof, can be produced as described above using the polynucleotides (or fragments or variants thereof) described in SEQ ID NO:3 and SEQ 30 ID NO:5, respectively. Rat and herpes CTLA-8 may also be produced as described in Rouvier et al., J. Immunol. 1993, 150, 5445-5456. CTLA-8 proteins of other

species can also be used as described herein. cDNAs encoding rat CTLA-8 and herpes CTLA-8 were deposited with the American Type Culture Collection on July 6, 1995 and assigned accession numbers ATCC 69867 and ATCC 69866, respectively. IL-17 proteins may also be produced as described in Golstein et al., *supra*.

5 Because of its homology to IL-17, the human CTLA-8 (B18) proteins of the present invention may also share some activities with IL-17.

For the purposes of treatment or therapy, any of the proteins discussed or disclosed herein may be administered by *in vivo* expression of the protein in a mammalian subject. In such instances, a polynucleotide encoding the desired protein 10 is administered to the subject in manner allowing expression in accordance with known methods, including without limitation the adenovirus methods disclosed herein.

Example 1

Isolation of Human CTLA-8 cDNA

A partial clone for human CTLA-8 was isolated from a cDNA library made from RNA isolated from stimulated human peripheral blood mononuclear cells. This partial was identified as "B18." B18 is sometimes used herein to refer to the human CTLA-8 of the present invention. Homology searches identified this partial clone as 20 being related to the herpes and rat CTLA-8 genes. DNA sequence of this partial clone was used to isolate the full-length clone.

In order to isolate a full-length cDNA for B18, a directional, full-length cDNA library by standard means in the COS expression vector pMV2. The cDNA library was transformed into *E. coli* by electroporation. The bulk of the original transformed 25 cDNA library was frozen in glycerol at -80°C. An aliquot was titered to measure the concentration of transformed *E. coli*. The *E. coli* were thawed, diluted to 76,000/0.1 ml in media containing ampicillin, and 0.1 ml was distributed into the wells of a microtiter dish in an 8 x 8 array. The microtiter dish was placed at 37°C overnight to grow the *E. coli*.

30 To prepare DNA for PCR, 20 µl aliquots of culture from each well were withdrawn and pooled separately for each row and column of eight wells, giving 16

pools of 160 µl each. The *E. coli* were pelleted, resuspended in 160 µl of standard lysis buffer consisting of 10 mM TrisHCl pH8, 1 mM EDTA, 0.01% Triton X-100, and lysed by heating to 95°C for 10 minutes.

5 To identify which of the wells contained *E. coli* transformed with B18, PCR was performed first on the DNA preps corresponding to the eight columns. The PCR consisted of two sequential reactions with nested oligonucleotides using standard conditions. The oligonucleotides used for the PCR reaction were derived from the sequence of the partial B18 clone. They were:

10 B185: CACAGGCATACACAGGAAGATACTTCA (SEQ ID NO:7)
 B183: TCTTGCTGGATGGAACCGGAATTCA (SEQ ID NO:8)
 B18N: ATACATTCACAGAAGAGCTCCTGCACA (SEQ ID NO:9)

15 The PCR conditions were 2.5 mM MgCl₂ and 95°C x 2 min for one cycle, 95°C x 1 min plus 68°C x 1 min for 30 cycles, and 68°C x 10 for one cycle. Each reaction was 20 µl. The first reaction contained oligonucleotides B185 and B183 and 1µl of the DNA preparations. The second reaction contained oligonucleotides B183 and B18N and 1 µl of the first reaction.

20 DNA preps that potentially contained a full-length B18 cDNA clone were identified by agarose gel electrophoresis on an aliquot of the second PCR reaction. A DNA band of the correct mobility was assumed to be derived from a B18 cDNA. Next the same sequence of PCR reactions and gel analysis was done on the DNA preps corresponding to the eight rows. The intersection of a row and a column identified well A2 as potentially containing B18, narrowing it down to the 76,000 *E. coli* originally seeded into that well.

25 To further purify the individual *E. coli* containing the putative full-length B18 cDNA clone, the concentration of *E. coli* in well A2 was measured by titering and plating dilutions of the well. Then 7600 *E. coli* were seeded into the wells of a second microtiter plate in an 8 x 8 array. The *E. coli* were grown overnight; wells were pooled, and DNA was prepared as described above. To identify which of these wells contained *E. coli* transformed with B18, sequential PCR reactions were performed

essentially as described above. Agarose gel electrophoresis identified well B2 as potentially containing a B18 cDNA.

The *E. coli* containing this cDNA was further purified by seeding wells of a microtiter plate with 253 *E. coli* per well and proceeding as for the purification of the 5 *E. coli* in well A2. Well C3 was identified as containing a putative full-length B18 cDNA clone. The exact *E. coli* was identified by plating the contents of the well onto bacterial culture media and then screening the *E. coli* colonies following established protocols. The probe for these hybridizations was a PCR fragment generated by doing a PCR reaction on the B18 clone using as primers the oligonucleotides described 10 above (SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9). When a single colony was identified, DNA was prepared and sequenced by standard methods. Comparison of this sequence to the sequence of the original partial clone confirmed identity and that the isolated cDNA was full-length.

The full-length clone was deposited with the American Type Culture 15 Collection on July 6, 1995 and assigned accession number ATCC 69868.

Example 2

Expression of Human CTLA-8

The full-length B18 clone for human CTLA-8 was transfected into COS cells 20 which were then labelled with ^{35}S -methionine. An aliquot of conditioned medium from the transfected cell culture was reduced, denatured and electrophoresed on polyacrylamide gels. Autoradiographs of those gels are reproduced in Fig. 2. The band indicated by the arrow demonstrates expression of human CTLA-8.

25

Example 3

Inhibition of Angiogenesis by Human CTLA-8

The ability of human CTLA-8 to inhibit angiogenesis was examined in an angiostatic activity assay (endothelial cell proliferation assay). The assay was done in a 96 well plate. Primary human umbilical cells (HUEVCs) were seeded to 2×10^3 30 cells per well in EGM medium (Clonetics)/20% FCS and incubated at 37°C for 24 hr. The cells were then starved in M199 medium (GIBCO BRL) containing 10% charcoal

treated serum (M199-CS) for 48 hr at 37°C. Conditioned media containing B18 (human CTLA-8) was obtained from transfected COS or stably expressing CHO cells and 1:10, 1:50, 1:250, and 1:1250 dilutions prepared in M199-CS medium containing 100 ng/ml FGF. The dilutions of B18 were added to the starved cells and incubated 5 for 72 hr at 37°C. The cells were then radiolabeled by [³H]-thymidine for 6 hr. Radiolabeled cells were washed with PBS and trypsinized for liquid scintillation counting. Results were plotted using Kaleidograph software. The results are shown in Fig. 3. In the figure, "Med" is the mock control, "B18" and "B18-1" were conditioned medium from two independent transfections of COS with DNA encoding 10 human CTLA-8 (B18). IFN γ was used as a positive control angiostatic (i.e., angiogenesis inhibition) activity. These data demonstrate that human CTLA-8 (B18) inhibits angiogenesis.

Example 4

15 Hematopoietic Activity of Human CTLA-8

The hematopoietic activity of human CTLA-8 (B18) expressed *in vivo* was examined by construction of a recombinant adenovirus vector.

The B18 cDNA in the expression plasmid Adori 2-12 B18 was driven by the cytomegalovirus(CMV) immediate early promoter and enhancer.

20 The Adori 2-12 vector was created by addition of an SV40 origin and enhancer to a known adenovirus vector (Barr et al., Gene Therapy 1:51 (1994); Davidson et al., Nature Genetics 3:219 (1993)). The HindIII/BamHI fragment encoding the SV40 origin and enhancer was isolated from the pMT2 mammalian expression vector, blunted with Klenow and cloned into the NatI site (blunted with Klenow) of the Ad5 25 expression vector.

The vector was derived by digesting pNOT-B18 cDNA with *SaII*, filling in the 5' overhang with Klenow to generate a blunt end and digesting with *EcoRI* to isolate the B18 cDNA. The blunted- *EcoRI* B18 fragment was inserted into the restriction sites *EcoRV-EcoRI* of the adenovirus vector Adori 2-12. The CMV-B18 expression cassette was located downstream of the SV40 origin and enhancer, and 0-1 map units 30 of the left hand end of the adenovirus type 5(Ad5). The SV40 splice donor and

acceptor were located between the CMV promoter and B18 cDNA. Following the insert was SV40 poly A site, 9-16 map units of Ad5 and the puc 19 origin.

A recombinant adenovirus was generated by homologous recombination in 293 cells. *Ascl* linearized Adori 2-12 B18 and *Clal* digested AdCMVlacZ were introduced 5 into the 293 cells using lipofectamine. Recombinant adenovirus virus was isolated and amplified on 293 cells. The virus was released from infected 293 cells by three cycles of freeze-thawing. The virus was further purified by two cesium chloride centrifugation gradients and dialyzed against PBS 4°C. Following dialysis of the virus glycerol was added to a concentration of 10 % and the virus was stored at - 70 °C until 10 use. The virus was characterized by expression of the transgene, plaque forming units on 293 cells, particles/ml and Southern analysis of the virus.

A single dose of 5×10^{10} particles of recombinant adenovirus encoding B18 was injected into the tail vein of male C57/bl6 mice, age 7-8 weeks. Control mice received an adenovirus encoding B-galactosidase. Four mice from each experimental 15 group were killed on day 7 and 14. Blood was collected and automated hematologic analysis was performed using a Baker 9000. Differential counts were performed on blood smears. Tissue was harvested, fixed in formalin, and stained with hematoxylin and eosin for histopathology. In the first set of experiments, serum and tissues were analyzed 7 and 14 days post injection. A slight increase in peripheral platelet counts 20 were observed. The animals that received B18 exhibited a slight increase in spleen size. Macroscopic analysis of the spleen showed an increase in splenic extramedullary hematopoiesis on day 7 compared to the control. These results showed a hematopoietic growth activity associated with B18.

In a second set of experiments 5×10^{10} particles of recombinant adenovirus 25 encoding B18 were injected into the tail vein of male C57/bl6 mice, age 17-18 weeks. Control mice received an adenovirus encoding B-galactosidase. Blood samples were collected via retro-orbital sinus on days 2, 5, 7, 10, 14, and 21. The hematologic analyses were performed on the Baker 9000 automated cell counter with murine-specific settings. Analyses included WBC, RBC, HCT and PLT counts. Blood smears 30 were prepared and stained with Wright-Geimsa for WBC differentials based on a 100 cellcount. Reticulocytes and reticulated platelets were quantitated using flow

cytometry. Four mice from each group were killed on days 7, 14, and 21. In addition to peripheral blood analysis, serum was collected via cardiac puncture for quantitation of systemic IL-6 using a commercial kit (Endogen). Spleen and liver were collected for histopathology, spleen and bone marrow hematopoietic progenitors were quantitated, 5 and bone marrow smears were prepared and stained with Wright-Geimsa for cell counts.

Administration of adenovirus encoding B18 resulted in a marked increase in peripheral blood neutrophils and WBC (Fig. 4). Maximum increases in neutrophils were observed at day 5 and day 7. The control mice showed little difference at day 5 10 and day 7. Peripheral blood neutrophils were similar in the control mice and mice that received B18 at day 21. In both the B18 and control groups an increase in white blood cells was also observed. The mice that received B18 had a greater increase in WBC between day 2 and day 7. By Day 21 a more pronounced increase was observed in the B-gal group. No other changes in cellular chemistries were observed (Table I).

15 Bone marrow cellularity was calculated from pooled femurs in each group (Table III). No significant differences were observed in either group. No significant changes were observed in bone marrow hematopoietic progenitors from day 7, 14, and 21. The CFU-GM, BFU-E and CFU-MEG in the B18 mice were similar to the B-gal control (Table II).

20 Administration of the adenovirus encoding B18 resulted in an increase in CFU-GM (myeloid) and BFU-E (erythroid) progenitors in the spleen compared to animals that received the B-gal virus on day 7. The increase in progenitors in the B18 mice was 11-fold in CFU-GM and a 52-fold in BFU-E (Table II). There was a 2-fold increase in CFU-MEG at day 7 for the B18 mice. By day 21 no significant differences 25 were observed in splenic CFU-MEG or BFU-E between the groups (Table II). A 3-fold decrease in CFU-GM was observed in mice that received adenovirus encoding B18. A slight increase in spleen size at day 7 was observed in the B18 group. This is consistent with an increase in splenic cellularity. By day 14 and day 21 spleen weights were similar to the control group (Table III). Macroscopic analysis of the 30 spleen showed an increase in splenic extramedullary hematopoiesis of the B18 mice on day 7 compared to the control.

The bone marrow myeloid: erythroid ratios (Table IV) suggest a granulocytic hyperplasia with a possible erythroid hypoplasia in mice that received adenovirus B18 on day 7. By day 21 the ratio in the B-gal group was higher. No changes were observed in the IL6 serum levels.

5 These results show a hematopoietic activity associated with the administration of adenovirus encoding B18 (human CTLA-8). Increases in neutrophils and white blood cells were observed at day 7 in animals that received B18 adenovirus. The data showed that B18 resulted in increase in splenic CFU-GM and BFU-E 7 days post administration compared to the control animals. Splenic extramedullary hematopoiesis on day 7 support that B18 exhibits a hematopoietic growth activity.
10 These data suggest that B18 may mobilize early hematopoietic precursors.

Table I: Peripheral hematology for day 2, 5, 7, 10, 14, and 21.

Study A54-4B...B18 (Platelets) Day 2...4-25-96.														
Group A	WBC x10 ³ /μL	Neuts %	ANC x10 ³ /μL	Lymphs %	ALC x10 ³ /μL	Eos %	Monos %	RBC x10 ⁶ /μL	Retics %	Abs Retics x10 ⁶ /μL	HCT %	PLT x10 ³ /μL	RPR %	Abs RPR x10 ⁻³ /μL
B-Gel #1	5.4	40	2.16	54	2.92	0	6	10.88	3.65	0.40	48.0	836	11.94	99.82
B-Gel #2	7.4	25	1.85	65	4.81	3	7	12.34	2.04	0.25	56.6	900	10.10	90.90
B-Gel #3	6.8	40	2.72	52	3.54	2	6	11.26	3.26	0.37	51.6	894	9.77	87.34
B-Gel #4	8.8	23	2.02	64	5.63	1	12	12.00	2.55	0.31	54.8	840	10.63	89.29
AVG	7.1	32.0	2.19	58.8	4.22	1.5	7.8	11.62	2.88	0.33	52.8	868	10.61	91.84
SEM	0.7	4.6	0.19	3.4	0.61	0.6	1.4	0.33	0.36	0.03	1.9	17	0.49	2.76
B18 #1	11.4	59	6.73	31	3.53	1	9	11.16	4.88	0.54	52.4	1242	14.92	185.31
B18 #2	9.2	30	2.76	62	5.70	3	5	10.14	3.97	0.40	46.0	632	10.90	66.89
B18 #3	5.0	51	2.55	40	2.00	0	8	11.16	3.23	0.36	51.2	832	11.18	93.02
B18 #4	6.4	41	2.62	55	3.52	0	4	10.80	3.09	0.33	49.2	904	17.31	156.46
AVG	8.0	45.3	3.67	47.0	3.65	1.0	6.8	10.82	3.79	0.41	50.2	903	13.58	125.92
SEM	1.4	6.3	1.02	7.0	0.76	0.7	1.3	0.24	0.41	0.05	1.0	127	1.55	27.07

Study A54-4B...B18 (Platelets) Day 5...4-26-96.														
Group B	WBC x10 ³ /μL	Neuts %	ANC x10 ³ /μL	Lymphs %	ALC x10 ³ /μL	Eos %	Monos %	RBC x10 ⁶ /μL	Retics %	Abs Retics x10 ⁶ /μL	HCT %	PLT x10 ³ /μL	RPR %	Abs RPR x10 ⁻³ /μL
B-Gel #1	7.8	14	1.06	78	5.83	3	5	11.26	5.25	0.59	52.4	1062	15.51	167.82
B-Gel #2	10.6	20	2.12	78	8.27	1	1	10.72	4.71	0.50	49.4	994	17.37	172.86
B-Gel #3	6.8	18	1.51	69	6.07	2	11	11.12	3.40	0.38	51.2	916	9.55	87.46
B-Gel #4	10.8	35	4.10	58	6.26	0	4	10.22	6.21	0.63	47.0	1092	13.93	152.12
AVG	9.5	22.5	2.20	70.8	6.63	1.5	5.3	10.83	4.89	0.63	50.0	1021	14.09	145.02
SEM	0.8	5.3	0.67	4.8	0.55	0.6	2.1	0.23	0.59	0.06	1.2	41	1.57	19.87
B18 #1	14.8	18	2.66	71	10.51	1	10	12.66	2.31	0.29	57.0	1204	7.57	91.14
B18 #2	14.2	37	5.25	53	7.53	2	6	9.80	3.32	0.33	44.6	886	14.33	127.25
B18 #3	12.8	30	3.64	69	7.55	1	10	12.12	4.12	0.50	53.6	1134	10.15	115.10
B18 #4	16.0	58	9.28	37	5.92	0	5	11.04	3.93	0.43	50.8	1166	15.75	183.85
AVG	14.5	35.8	5.26	65.0	7.85	1.0	8.3	11.41	3.42	0.39	52.0	1086	11.95	122.28
SEM	0.7	8.4	1.44	7.1	0.95	0.4	1.2	0.83	0.41	0.05	2.8	71	1.84	15.81

Study A54-4B...B18 (Platelets) Day 7...4-30-96.														
Group C	WBC x10 ³ /μL	Neuts %	ANC x10 ³ /μL	Lymphs %	ALC x10 ³ /μL	Eos %	Monos %	RBC x10 ⁶ /μL	Retics %	Abs Retics x10 ⁶ /μL	HCT %	PLT x10 ³ /μL	RPR %	Abs RPR x10 ⁻³ /μL
B-Gel #1	15.2	14	2.13	69	10.49	1	16	11.04	3.54	0.39	50.8	662	12.46	107.41
B-Gel #2	14.0	12	1.68	81	11.34	0	7	11.38	8.05	0.57	52.6	1104	14.91	164.81
B-Gel #3	14.8	14	2.07	73	10.80	1	12	10.92	5.42	0.59	49.8	952	11.49	109.28
B-Gel #4	14.7	13.3	1.86	74.3	10.88	0.7	11.7	11.11	4.87	0.52	61.0	973	12.95	127.13
AVG	17.5	3.0	1.64	73.5	10.88	0.3	2.6	10.14	0.58	0.06	59.9	71	1.02	18.75
SEM	0.4	0.7	0.14	3.5	0.25	0.3	1.1							
B18 #1	19.4	33	6.40	62	12.03	0	5	10.14	2.93	0.30	45.2	864	12.80	110.59
B18 #2	23.4	39	9.91	53	13.46	0	8	9.48	6.05	0.57	43.6	1288	12.49	180.87
B18 #3	23.8	44	10.38	50	11.80	0	6	9.74	5.17	0.50	44.4	1076	15.41	185.81
B18 #4	12.8	15	1.92	75	9.80	0	10	9.54	6.28	0.60	43.4	1136	15.80	180.40
AVG	19.8	32.8	7.15	60.0	11.72	0.0	7.3	9.72	6.10	0.49	44.2	1091	14.15	184.42
SEM	2.8	6.3	1.98	6.6	0.90	0.0	1.1	0.15	0.76	0.07	0.4	88	0.87	15.19

Study A54-4B...B18 (Platelets) Day 10...5-3-96.														
Group A	WBC x10 ³ /μL	Neuts %	ANC x10 ³ /μL	Lymphs %	ALC x10 ³ /μL	Eos %	Monos %	RBC x10 ⁶ /μL	Retics %	Abs Retics x10 ⁶ /μL	HCT %	PLT x10 ³ /μL	RPR %	Abs RPR x10 ⁻³ /μL
B-Gel #1	18.6	17	3.16	69	12.83	3	11	10.22	12.41	1.27	46.8	1460	16.20	236.52
B-Gel #2	13.2	16	2.11	79	10.43	1	4	10.46	6.00	0.63	48.8	1128	14.48	183.33
B-Gel #3	19.6	16	3.14	74	14.50	0	10	10.72	6.25	0.57	49.4	1338	18.58	221.84
B-Gel #4	18.6	21	3.31	72	13.39	3	4	10.44	7.59	0.70	48.4	1068	14.35	153.26
AVG	17.5	17.5	3.08	73.5	12.79	1.8	7.3	10.47	8.06	0.84	48.4	1249	15.40	193.74
SEM	1.5	1.2	0.37	2.1	0.88	0.8	1.1	0.10	1.49	0.15	0.5	91	0.58	20.78
B18 #1	14.2	33	4.69	56	7.95	5	6	8.70	11.57	1.04	38.2	1760	14.49	255.02
B18 #2	17.8	35	6.16	57	10.03	1	7	9.04	9.48	0.86	42.0	1104	16.86	206.44
B18 #3	16.2	39	6.32	57	9.23	1	3	9.47	16.77	0.79	22.4	834	20.19	260.96
B18 #4	14.2	25	3.55	66	8.37	1	8	9.30	9.93	0.92	42.0	1416	16.81	238.03
AVG	15.6	33.0	6.16	69.0	8.15	2.0	6.0	7.85	12.04	0.80	38.4	1294	19.84	246.61
SEM	0.8	2.9	0.66	2.3	0.43	1.0	1.1	1.05	1.87	0.05	4.7	183	3.24	11.77

Study A54-4B...B18 (Platelets) Day 21...5-14-96.														
Group A	WBC x10 ³ /μL	Neuts %	ANC x10 ³ /μL	Lymphs %	ALC x10 ³ /μL	Eos %	Monos %	RBC x10 ⁶ /μL	Retics %	Abs Retics x10 ⁶ /μL	HCT %	PLT x10 ³ /μL	RPR %	Abs RPR x10 ⁻³ /μL
B-Gel #1	25.4	23	5.84	67	17.02	0	10	9.22	8.15	0.75	42.8	1776	9.61	170.67
B-Gel #2	19.8	19	3.72	69	15.52	0	12	9.50	9.95	0.95	44.4	1682	9.44	158.89
B-Gel #3	27.6	11	3.04	82	22.63	3	4	9.74	8.84	0.86	45.8	1684	11.45	182.82
B-Gel #4	26.0	13	3.64	83	22.24	0	4	9.04	7.54	0.68	41.6	1346	10.48	141.06
AVG	25.2	16.5	4.06	75.3	19.10	0.8	7.5	9.38	8.62	0.81	43.7	1617	10.25	165.36
SEM	1.9	2.8	0.61	4.2	2.33	0.8								

Table II: Bone marrow and Splenic Hematopoietic Progenitors

	CFU-MEG		CFU-GM		BFU-E		
	Bone Marrow*	B-Gal	B18	B-Gal	B18	B-Gal	B18
5	Day 7	16.0 ± 3.5	15.7 ± 3.1	307 ± 117	241 ± 78	51 ± 19	25 ± 11
	Day 14	10.7 ± 2.3	15.3 ± 1.2	233 ± 15	373 ± 35	30 ± 10	60 ± 30
	Day 21	5.7 ± 0.6	6.7 ± 3.1	170 ± 17	160 ± 27	40 ± 10	27 ± 6
	Spleen**						
10	Day 7	9.3 ± 1.6	19.5 ± 1.5	27 ± 3	298 ± 6	1.3 ± 1.2	68 ± 10
	Day 14	9.7 ± 0.6	12.7 ± 0.6	267 ± 32	197 ± 21	33 ± 6	10 ± 10
	Day 21	17.0 ± 1.0	19.3 ± 2.5	187 ± 6	73 ± 15	23 ± 6	23 ± 6

Hematopoietic precursors were determined from pooled spleen and bone marrow samples from four animals in each group. For quantitation of CFU-GM and BFU-E, either 1×10^4 bone marrow cells or 1×10^5 spleen cells were added to complete alpha methylcellulose medium (0.9% methylcellulose in alpha medium, 30% fetal bovine serum, 1% bovine serum albumin, 10-4M 2-mercaptoethanol, 2 mM L-glutamine, 2% murine spleen cell conditioned medium, and 3 U/mL erythropoietin) and aliquoted into 35 mm tissue culture dishes in a final volume of 1.0 mL. Cultures were incubated for 7 days at 37°C, and 5% CO₂. Microscopic colonies were defined as clusters of 50 or more cells. For quantitation of CFU-MEG, either 1×10^5 bone marrow cells or 1×10^6 spleen cells were added to complete alpha methylcellulose medium and incubated as described above. Megakaryocyte colonies were defined as a group of 3 or more cells.

*Bone marrow progenitors are represented as mean ± sd number of colonies per 10^5 cells.

**Spleen progenitors are represented as mean ± sd number of colonies per 10^6 cells.

Table III: Spleen Weights and Femur Cellularity

Spleen Wt. (Mg)	B-Gal	B18		Femur Cellularity ($\times 10^6$)	B-Gal	B18
Day 7	187 ± 19	224 ± 29		Day 7	28	23
Day 14	175 ± 13	170 ± 10		Day 14	28	27
Day 21	174 ± 21	151 ± 27		Day 21	28	26

5 Spleen weights were determined at time of sacrifice are represented as means ± sd from four animals.

10 Table IV: Bone Marrow Myeloid:Erythroid Ratios

Group	Mouse #	Day 7	Day 14	Day 21
B-gal	1	1.43	2.12	5.78
	2	0.91	2.46	5.83
	3	1.62	1.03	3.66
	4		5.44	4.82
AVG		1.32	2.76	5.02
SD		0.37	0.37	1.89
B-gal	1	5.59	2.01	2.02
	2	6.51	1.25	2.13
	3	5.49	1.58	1.81
	4	0.50	2.51	2.92
AVG		4.52	1.86	2.22
SD		1.29	2.72	0.56

15 20 25 All entries represent the number of myeloid cells per 1 erythroid cell. Normal mouse ratios are approximately 1:1 to 2:1.

Example 5

30 Additional Experiments Relating to

Hematopoietic Activity of Human CTLA-8

B18 (human CTLA-8) was tested for the ability to induce production of factors having hematopoietic activity in a factor-dependent cell proliferation assay using the

human erythroleukemic cell line, TF-1 (Kitamura et al., J. Cell Physiol. 140:323 (1989)). The cells were initially grown in the presence of rhGMCSF (100 U/ml). The cells were fed three days prior to setting up the assay. The assay conditions were as follows:

5	cells/well	5000/200μl
	incubation time	3 days
	pulse time	4 hours
	amount of tritiated thymidine	0.5μCi/well
	counting time	1 minute
10	replicates	2

B18 alone, conditioned medium (CM) from B18 induced HS-5 cells were assayed. Buffer alone, CM from HS-5 cells induced with buffer and CM from uninduced HS-5 cells were assayed as controls. Results are shown in Fig. 5. B18 (human CTLA-8) demonstrated an ability to induce production of factors which induced TF-1 proliferation. This activity was substantially eliminated by the addition of anti-GMCSF antibodies. These data demonstrate that human CTLA-8 (B18) is able to induce hematopoiesis. Particularly, without being bound by any theory, it appears that human CTLA-8 (B18) induces production of GM-CSF and/or IL-3.

20

Example 6

Ability of Human CTLA-8 to Induce Production of IL-6 and IL-8

MRC5 cells were incubated in the presence of human CTLA-8 (B18) and production of IL-6 and IL-8 were measured. Herpes CTLA-8 (IL-17) was used as a positive control. Applicants' human CTLA-8 (B18) demonstrated titratable production of both IL-6 and IL-8 (see Figs. 6 and 7).

All patent and literature references cited herein are incorporated by reference as if fully set forth.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Human CTLA-8 and Uses of CTLA-8-Related Proteins

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

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(E) COUNTRY: USA
(F) ZIP: 02140

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Brown, Scott A.
(B) REGISTRATION NUMBER: 32,724
(C) REFERENCE/DOCKET NUMBER: GI5262

(ix) TELECOMMUNICATION INFORMATION:

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(B) TELEFAX: (617) 876-5851

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 813 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 56..544

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGAAGATAC ATTACAGAA AGAGCTTCCT GCACAAAGTA AGCCACCAGC GCAAC ATG	58
Met	
1	
ACA GTG AAG ACC CTG CAT GCC CCA GCC ATG GTC AAG TAC TTG CTG CTG	106
Thr Val Lys Thr Leu His Gly Pro Ala Met Val Lys Tyr Leu Leu Leu	
5 10 15	
TCG ATA TTG GGG CTT GCC TTT CTG AGT GAG GCG GCA GCT CGG AAA ATC	154
Ser Ile Leu Gly Leu Ala Phe Leu Ser Glu Ala Ala Ala Arg Lys Ile	
20 25 30	
CCC AAA GTA GGA CAT ACT TTT TTC CAA AAG CCT GAG AGT TGC CCG CCT	202
Pro Lys Val Gly His Thr Phe Phe Gln Lys Pro Glu Ser Cys Pro Pro	
35 40 45	
GTG CCA GGA GGT AGT ATG AAG CTT GAC ATT GGC ATC ATC AAT GAA AAC	250
Val Pro Gly Gly Ser Met Lys Leu Asp Ile Gly Ile Ile Asn Glu Asn	
50 55 60 65	
CAG CGC GTT TCC ATG TCA CGT AAC ATC GAG AGC CGC TCC ACC TCC CCC	298
Gln Arg Val Ser Met Ser Arg Asn Ile Glu Ser Arg Ser Thr Ser Pro	
70 75 80	
TGG AAT TAC ACT GTC ACT TGG GAC CCC AAC CGG TAC CCC TCG GAA GTT	346
Trp Asn Tyr Thr Val Thr Trp Asp Pro Asn Arg Tyr Pro Ser Glu Val	
85 90 95	
GTA CAG GCC CAG TGT AGG AAC TTG GGC TGC ATC AAT GCT CAA GGA AAG	394
Val Gln Ala Gln Cys Arg Asn Leu Gly Cys Ile Asn Ala Gln Gly Lys	
100 105 110	
GAA GAC ATC TCC ATG AAT TCC GTT CCC ATC CAG CAA GAG ACC CTG GTC	442
Glu Asp Ile Ser Met Asn Ser Val Pro Ile Gln Gln Glu Thr Leu Val	
115 120 125	
GTC CGG AGG AAG CAC CAA GGC TGC TCT GTT TCT TTC CAG TTG GAG AAG	490
Val Arg Arg Lys His Gln Gly Cys Ser Val Ser Phe Gln Leu Glu Lys	
130 135 140 145	
GTG CTG GTG ACT GTT GGC TGC ACC TGC GTC ACC CCT GTC ATC CAC CAT	538
Val Leu Val Thr Val Gly Cys Thr Cys Val Thr Pro Val Ile His His	
150 155 160	
GTG CAG TAAGAGGTGC ATATCCACTC AGCTGAAGAA GCTGTAGAAA TGCCACTCCT	594
Val Gln	
TACCCAGTGC TCTGCAACAA GTCCTGTCTG ACCCCCCAATT CCCTCCACTT CACAGGACTC	654
TTAATAAGAC CTGCACGGAT GGAAACAGAA AATATTCA CA ATGTATGTGT GTATGTACTA	714
CACTTTATAT TTGATATCTA AAATGTTAGG AGAAAAATTA ATATATTCA G TGCTAATATA	774
ATAAAAGTATT AATAATTAA AAATAAAAAA AAAAAAAA	813

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 163 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Val Lys Thr Leu His Gly Pro Ala Met Val Lys Tyr Leu Leu
 1 5 10 15

Leu Ser Ile Leu Gly Leu Ala Phe Leu Ser Glu Ala Ala Ala Arg Lys
 20 25 30

Ile Pro Lys Val Gly His Thr Phe Phe Gln Lys Pro Glu Ser Cys Pro
 35 40 45

Pro Val Pro Gly Gly Ser Met Lys Leu Asp Ile Gly Ile Ile Asn Glu
 50 55 60

Asn Gln Arg Val Ser Met Ser Arg Asn Ile Glu Ser Arg Ser Thr Ser
 65 70 75 80

Pro Trp Asn Tyr Thr Val Thr Trp Asp Pro Asn Arg Tyr Pro Ser Glu
 85 90 95

Val Val Gln Ala Gln Cys Arg Asn Leu Gly Cys Ile Asn Ala Gln Gly
 100 105 110

Lys Glu Asp Ile Ser Met Asn Ser Val Pro Ile Gln Gln Glu Thr Leu
 115 120 125

Val Val Arg Arg Lys His Gln Gly Cys Ser Val Ser Phe Gln Leu Glu
 130 135 140

Lys Val Leu Val Thr Val Gly Cys Thr Cys Val Thr Pro Val Ile His
 145 150 155 160

His Val Gln

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 461 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 6..455

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCACC ATG TGC CTG ATG CTG TTG CTG CTA CTG AAC CTG GAG GCT ACA 47
 Met Cys Leu Met Leu Leu Leu Leu Asn Leu Glu Ala Thr
 1 5 10

GTG AAG GCA GCG GTA CTC ATC CCT CAA AGT TCA GTG TGT CCA AAC GCC 95
 Val Lys Ala Ala Val Leu Ile Pro Gln Ser Ser Val Cys Pro Asn Ala
 15 20 25 30

GAG GCC AAT AAC TTT CTC CAG AAC GTG AAG GTC AAC CTG AAA GTC ATC 143
 Glu Ala Asn Asn Phe Leu Gln Asn Val Lys Val Asn Leu Lys Val Ile
 35 40 45

AAC TCC CTT AGC TCA AAA GCG AGC TCG AGA AGG CCC TCA GAT TAC CTC Asn Ser Leu Ser Ser Lys Ala Ser Ser Arg Arg Pro Ser Asp Tyr Leu 50 55 60	191
AAC CGT TCC ACT TCA CCC TGG ACT CTG AGC CGC AAT GAG GAC CCT GAT Asn Arg Ser Thr Ser Pro Trp Thr Leu Ser Arg Asn Glu Asp Pro Asp 65 70 75	239
AGA TAT CCT TCT GTG ATC TGG GAG GCA CAG TGC CGC CAC CAG CGC TGT Arg Tyr Pro Ser Val Ile Trp Glu Ala Gln Cys Arg His Gln Arg Cys 80 85 90	287
GTC AAC GCT GAG GGG AAG TTG GAC CAC CAC ATG AAT TCT GTT CTC ATC Val Asn Ala Glu Gly Lys Leu Asp His His Met Asn Ser Val Leu Ile 95 100 105 110	335
CAG CAA GAG ATA CTA GTC CTG AAG AGG GAG CCT GAG AAG TGC CCC TTC Gln Gln Glu Ile Leu Val Leu Lys Arg Glu Pro Glu Lys Cys Pro Phe 115 120 125	383
ACT TTC CGG GTG GAG AAG ATG CTG GTG GGC GTG GGC TGC ACC TGC GTT Thr Phe Arg Val Glu Lys Met Leu Val Gly Val Gly Cys Thr Cys Val 130 135 140	431
TCC TCT ATT GTC CGC CAT GCG TCC TAATAA Ser Ser Ile Val Arg His Ala Ser 145 150	461

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 150 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Cys Leu Met Leu Leu Leu Asn Leu Glu Ala Thr Val Lys 1 5 10 15
Ala Ala Val Leu Ile Pro Gln Ser Ser Val Cys Pro Asn Ala Glu Ala 20 25 30
Asn Asn Phe Leu Gln Asn Val Lys Val Asn Leu Lys Val Ile Asn Ser 35 40 45
Leu Ser Ser Lys Ala Ser Ser Arg Arg Pro Ser Asp Tyr Leu Asn Arg 50 55 60
Ser Thr Ser Pro Trp Thr Leu Ser Arg Asn Glu Asp Pro Asp Arg Tyr 65 70 75 80
Pro Ser Val Ile Trp Glu Ala Gln Cys Arg His Gln Arg Cys Val Asn 85 90 95
Ala Glu Gly Lys Leu Asp His His Met Asn Ser Val Leu Ile Gln Gln 100 105 110
Glu Ile Leu Val Leu Lys Arg Glu Pro Glu Lys Cys Pro Phe Thr Phe 115 120 125
Arg Val Glu Lys Met Leu Val Gly Val Gly Cys Thr Cys Val Ser Ser 130 135 140

Ile Val Arg His Ala Ser
145 150

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 459 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..453

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG ACA TTT AGA ATG ACT TCA CTT GTG TTA CTT CTG CTG CTG AGC ATA Met Thr Phe Arg Met Thr Ser Leu Val Leu Leu Leu Leu Ser Ile 1 5 10 15	48
GAT TGT ATA GTA AAG TCA GAA ATA ACT AGT GCA CAA ACC CCA AGA TGC Asp Cys Ile Val Lys Ser Glu Ile Thr Ser Ala Gln Thr Pro Arg Cys 20 25 30	96
TTA GCT GCT AAC AAT AGC TTT CCA CGG TCT GTG ATG GTT ACT TTG AGC Leu Ala Ala Asn Asn Ser Phe Pro Arg Ser Val Met Val Thr Leu Ser 35 40 45	144
ATC CGT AAC TGG AAT ACC AGT TCT AAA AGG GCT TCA GAC TAC TAC AAT Ile Arg Asn Trp Asn Thr Ser Ser Lys Arg Ala Ser Asp Tyr Tyr Asn 50 55 60	192
AGA TCT ACG TCT CCT TGG ACT CTC CAT CGC AAT GAA GAT CAA GAT AGA Arg Ser Thr Ser Pro Trp Thr Leu His Arg Asn Glu Asp Gln Asp Arg 65 70 75 80	240
TAT CCC TCT GTG ATT TGG GAA GCA AAG TGT CGC TAC TTA GGA TGT GTT Tyr Pro Ser Val Ile Trp Glu Ala Lys Cys Arg Tyr Leu Gly Cys Val 85 90 95	288
AAT GCT GAT GGG AAT GTA GAC TAC CAC ATG AAC TCA GTC CCT ATC CAA Asn Ala Asp Gly Asn Val Asp Tyr His Met Asn Ser Val Pro Ile Gln 100 105 110	336
CAA GAG ATT CTA GTG GTG CGC AAA GGG CAT CAA CCC TGC CCT AAT TCA Gln Glu Ile Leu Val Val Arg Lys Gly His Gln Pro Cys Pro Asn Ser 115 120 125	384
TTT AGG CTA GAG AAG ATG CTA GTG ACT GTA GGC TGC ACA TGC GTT ACT Phe Arg Leu Glu Lys Met Leu Val Thr Val Gly Cys Thr Cys Val Thr 130 135 140	432
CCC ATT GTT CAC AAT GTA GAC TAAAAG Pro Ile Val His Asn Val Asp 145 150	459

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 151 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Thr Phe Arg Met Thr Ser Leu Val Leu Leu Leu Leu Ser Ile
 1           5           10          15

Asp Cys Ile Val Lys Ser Glu Ile Thr Ser Ala Gln Thr Pro Arg Cys
 20          25          30

Leu Ala Ala Asn Asn Ser Phe Pro Arg Ser Val Met Val Thr Leu Ser
 35          40          45

Ile Arg Asn Trp Asn Thr Ser Ser Lys Arg Ala Ser Asp Tyr Tyr Asn
 50          55          60

Arg Ser Thr Ser Pro Trp Thr Leu His Arg Asn Glu Asp Gln Asp Arg
 65          70          75          80

Tyr Pro Ser Val Ile Trp Glu Ala Lys Cys Arg Tyr Leu Gly Cys Val
 85          90          95

Asn Ala Asp Gly Asn Val Asp Tyr His Met Asn Ser Val Pro Ile Gln
100         105         110

Gln Glu Ile Leu Val Val Arg Lys Gly His Gln Pro Cys Pro Asn Ser
115         120         125

Phe Arg Leu Glu Lys Met Leu Val Thr Val Gly Cys Thr Cys Val Thr
130         135         140

Pro Ile Val His Asn Val Asp
145         150

```

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CACAGGCATA CACAGGAAGA TACATTCA

28

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCTTGCTGGA TGGGAACCGGA ATTCA

25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATACATTCAC AGAAGAGCTT CCTGCACA

28

5 What is claimed is:

1. An isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 146 to nucleotide 544;

10 (b) a nucleotide sequence capable of hybridizing to a nucleic acid sequence specified in (a);

(c) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code; and

(d) an allelic variant of the nucleotide sequence specified in (a).

15

2. The polynucleotide of claim 1 wherein said nucleotide sequence encodes a protein having CTLA-8 activity.

20 3. The polynucleotide of claim 1 wherein said nucleotide sequence is operably linked to an expression control sequence.

4. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 55 to nucleotide 544.

25 5. A host cell transformed with the polynucleotide of claim 3.

6. The host cell of claim 5, wherein said cell is a mammalian cell.

7. A process for producing a human CTLA-8 protein, said process comprising:

30 (a) growing a culture of the host cell of claim 5 in a suitable culture medium; and

(b) purifying the human CTLA-8 protein from the culture.

5 8. An isolated human CTLA-8 protein comprising an amino acid sequence selected
from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;
(b) the amino acid sequence of SEQ ID NO:2 from amino acids 11 to 163;
(c) the amino acid sequence of SEQ ID NO:2 from amino acids 29 to 163;
10 (d) the amino acid sequence of SEQ ID NO:2 from amino acids 31 to 163; and
(e) fragments of (a), (b), (c) or (d) having CTLA-8 activity.

9. The protein of claim 8 comprising the amino acid sequence of SEQ ID NO:2.

15 10. The protein of claim 8 comprising the sequence from amino acid 29 to 163 of SEQ
ID NO:2.

11. A pharmaceutical composition comprising a human CTLA-8 protein of claim 8 and
a pharmaceutically acceptable carrier.

20 12. A human CTLA-8 protein produced according to the process of claim 7.

13. A composition comprising an antibody which specifically reacts with a human
CTLA-8 protein of claim 8.

25 14. A method of treating a mammalian subject comprising administering a
therapeutically effective amount of a composition of claim 11.

30 15. A method of treating a mammalian subject comprising administering a
therapeutically effective amount of a composition comprising a pharmaceutically
acceptable carrier and a protein comprising an amino acid sequence selected from the group
consisting of:

(a) the amino acid sequence of SEQ ID NO:4;

5 (b) the amino acid sequence of SEQ ID NO:4 from amino acids 18 to 150; and
 (c) fragments of (a) or (b) having CTLA-8 activity.

16. The method of claim 15 wherein said protein comprises the amino acid sequence
of SEQ ID NO:4.

1.0

17. The method of claim 15 wherein said protein comprises the amino acid sequence
of SEQ ID NO:4 from amino acids 18 to 150.

15

18. A method of treating a mammalian subject administering a therapeutically effective
amount of a composition comprising a pharmaceutically acceptable carrier and a protein
comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:6;
 (b) the amino acid sequence of SEQ ID NO:6 from amino acids 19 to 151; and
 (c) fragments of (a) or (b) having CTLA-8 activity.

20

19. The method of claim 18 wherein said protein comprises the amino acid sequence
of SEQ ID NO:6.

25

20. The method of claim 18 wherein said protein comprises the amino acid sequence
of SEQ ID NO:6 from amino acids 19 to 151.

21. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1
from nucleotide 86 to nucleotide 544.

30

22. The protein of claim 8 comprising the sequence from amino acid 11 to 163 of SEQ
ID NO:2.

5 23. A method of treating a mammalian subject comprising administering a therapeutically effective amount of a composition comprising a pharmaceutically acceptable carrier and IL-17 or an active fragment thereof.

10 24. The method of claim 14, 15, 18 or 23 wherein said subject is treated to produce an effect selected from the group consisting of inhibition of angiogenesis, inhibition of growth or proliferation of vascular endothelial cells, inhibition of tumor growth, inhibition of angiogenesis-dependent tissue growth, proliferation of myeloid cells or progenitors, proliferation of erythroid cells or progenitors, proliferation of lymphoid cells or progenitors, induction of IFN γ production, induction of IL-3 production and induction of GM-CSF production.

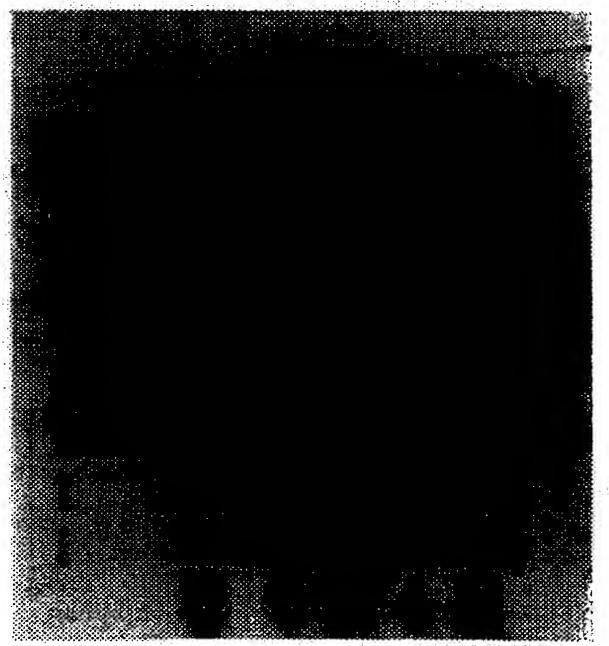
15 25. The composition of claim 3 wherein said polynucleotide is contained in a vector suitable for *in vivo* expression in a mammalian subject.

20 26. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 139 to nucleotide 544.

27. The protein of claim 8 comprising the sequence from amino acid 31 to 163 of SEQ ID NO:2.

Hsvie - 2	- - - [I]V[K]S B[IT]SAQ[T]PRCL-[A]ANN[S]P[R]SVW[V]T[L]S[I]RN	- - - [W]NT[S]SKR[A]SDYY[N]RSTSP	5 1
Musct1a8	- - - [A]V[L]PQ[-S]SVC[P]N[Q]AEANNE	[L]KVIN[S]SKASSR[R]PSDYL[N]RSTSP	5 1
B18 - F1	A AR K[I]P[K]PE S[C]P[V]PGG - - - [SM]KLDIGINENQ[R]VSM[SR]	- - NIE[S]RSTSP	5 3
Hsvie - 2	[WT]L[H]R[N]EDQ[D]RY[P]SSV[I]WEAK[C]RY[L]GC[V]NA[D]G[N]CDY[H]M[N]S[V]P[I]QQ[E]I[L]V[V]R[K]G[H]Q[P][C]P[N]SF	1 1 1	
Musct1a8	[WT]L[S]R[N]EDP[D]RY[P]SSV[I]WEAQ[C]R[H]Q[R]C[V]NA[E]G[K]LD[H]M[N]S[V]I[Q]Q[E]I[L]V[L]K[R]E[P]E[K]C[P]FT[F]	1 1 1	
B18 - F1	[W]NYT[V]T[W]D[P]N[R]Y[P]SE[V]VQAQCR[N]L[G]C[I]NA[Q]G[K]ED[DI]SM[N]S[V]P[I]QQ[E]I[L]V[V]R[R]K[H]Q[G]CS[V]SF	1 1 3	
Hsvie - 2	R[LE]K[M]L[V]T[V]G[C]T[C]V[T]P[I]V[H]N[V]D	1 3 3	
Musct1a8	R[LE]K[M]L[V]G[V]G[C]T[C]V[S]IV[R]HA	1 3 3	
B18 - F1	Q[LE]K[V]L[V]T[V]G[C]T[C]V[T]P[V]IHH[V]Q	1 3 5	

Fig. 1

Figure 2

200K—
97K—
69K—
46K—
30K—
23K—
14.3K—
→

B18 M B18 M
R R NR NR
Supernatant Cell Lysate
2/7

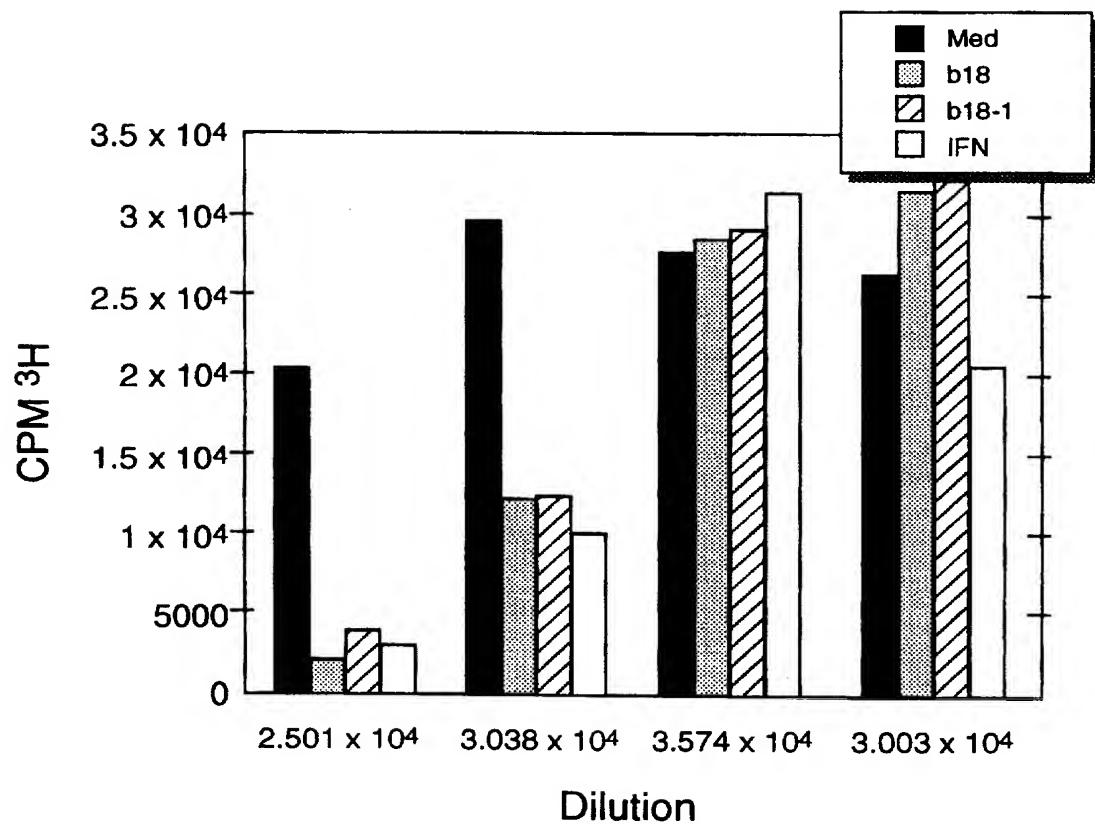


Fig. 3

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Fig. 4A

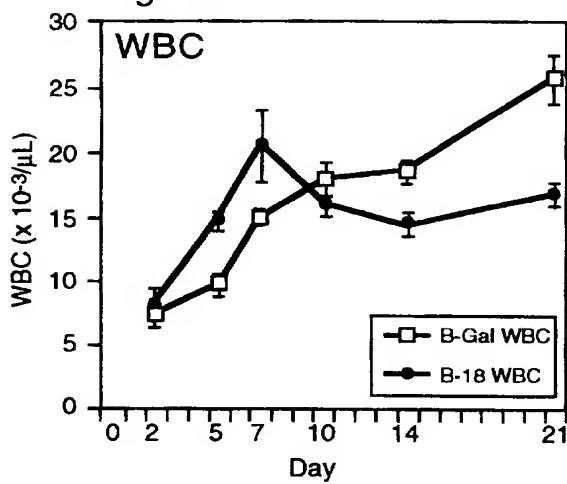


Fig. 4B

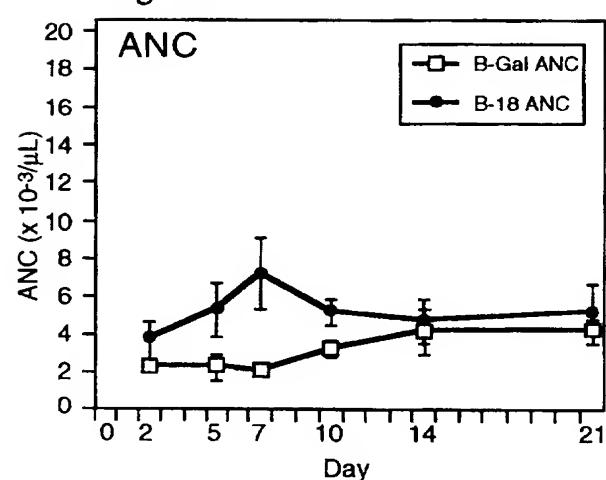


Fig. 4C

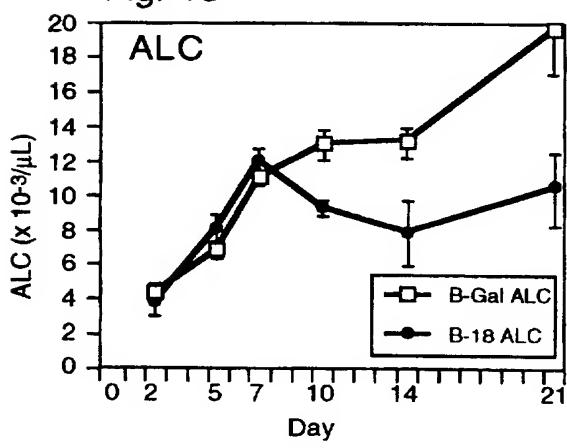
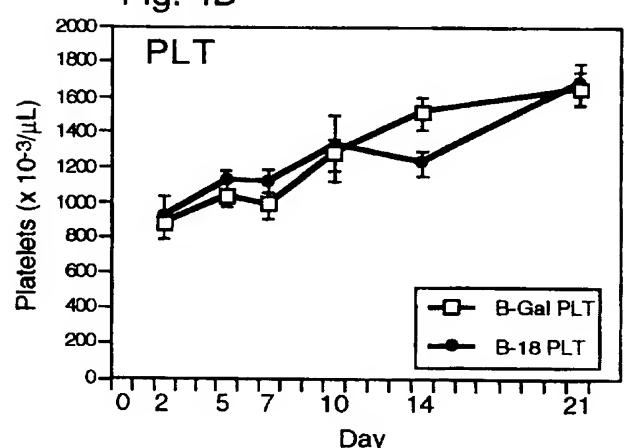


Fig. 4D



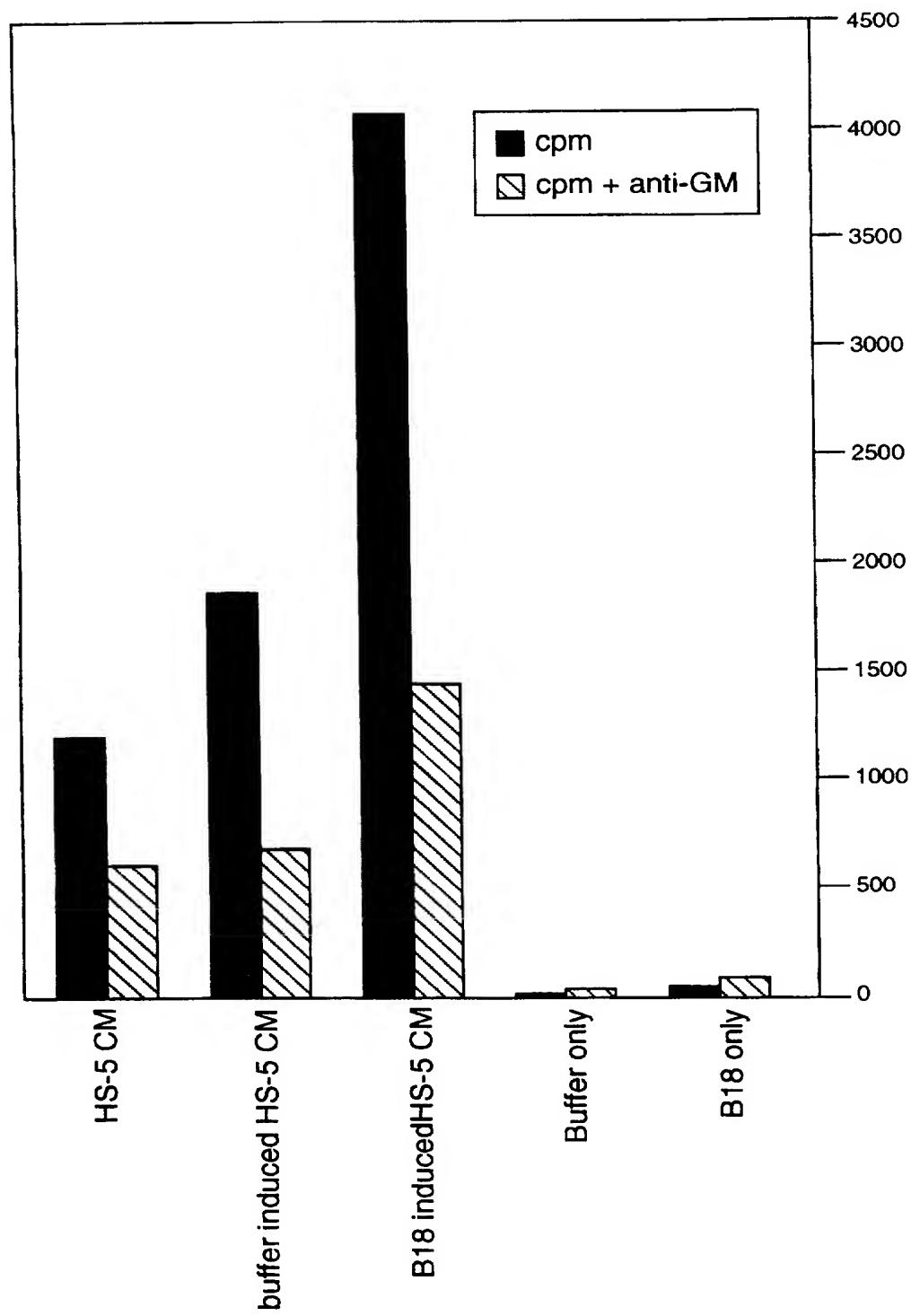


Fig. 5

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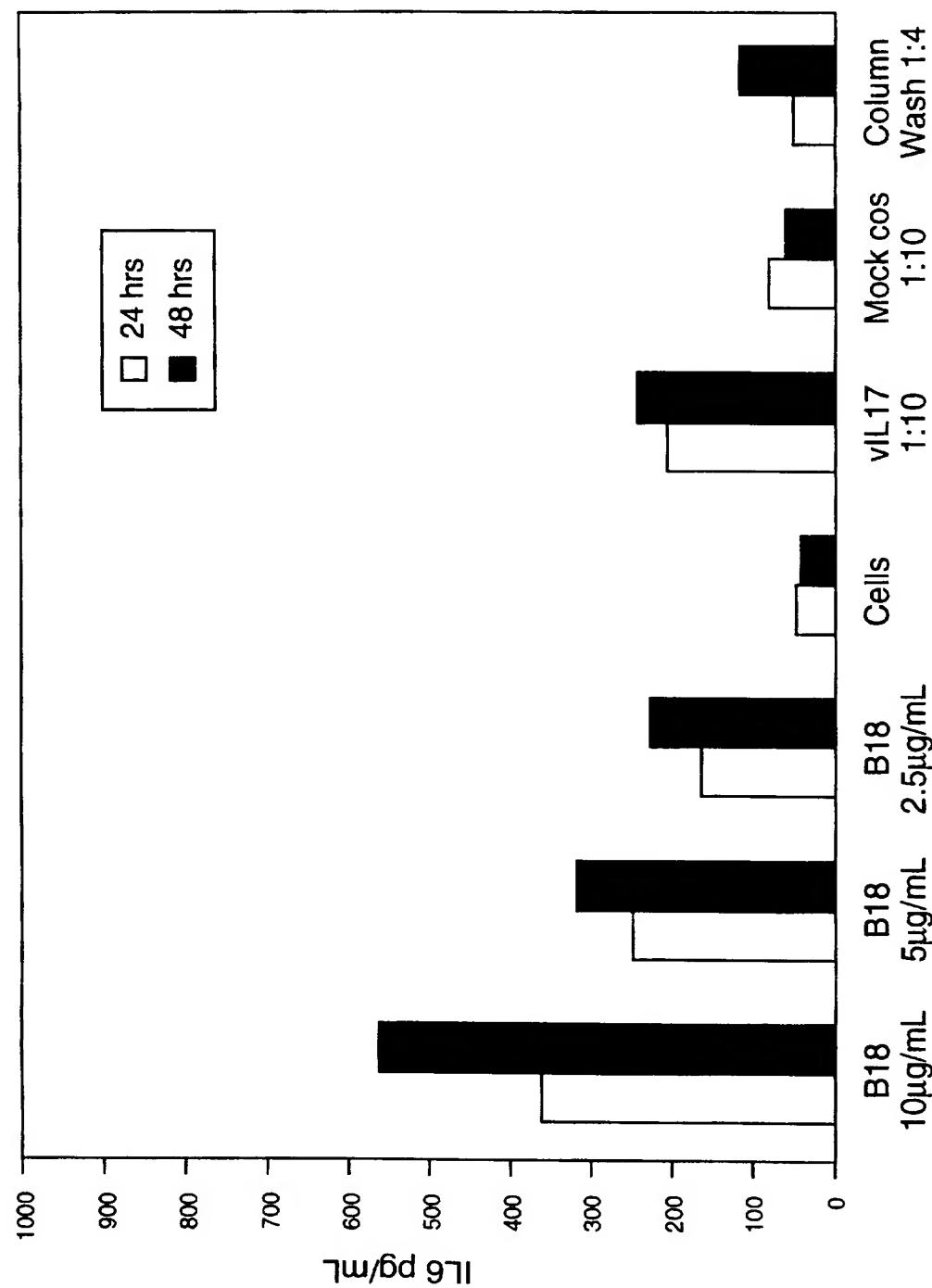


Fig. 6

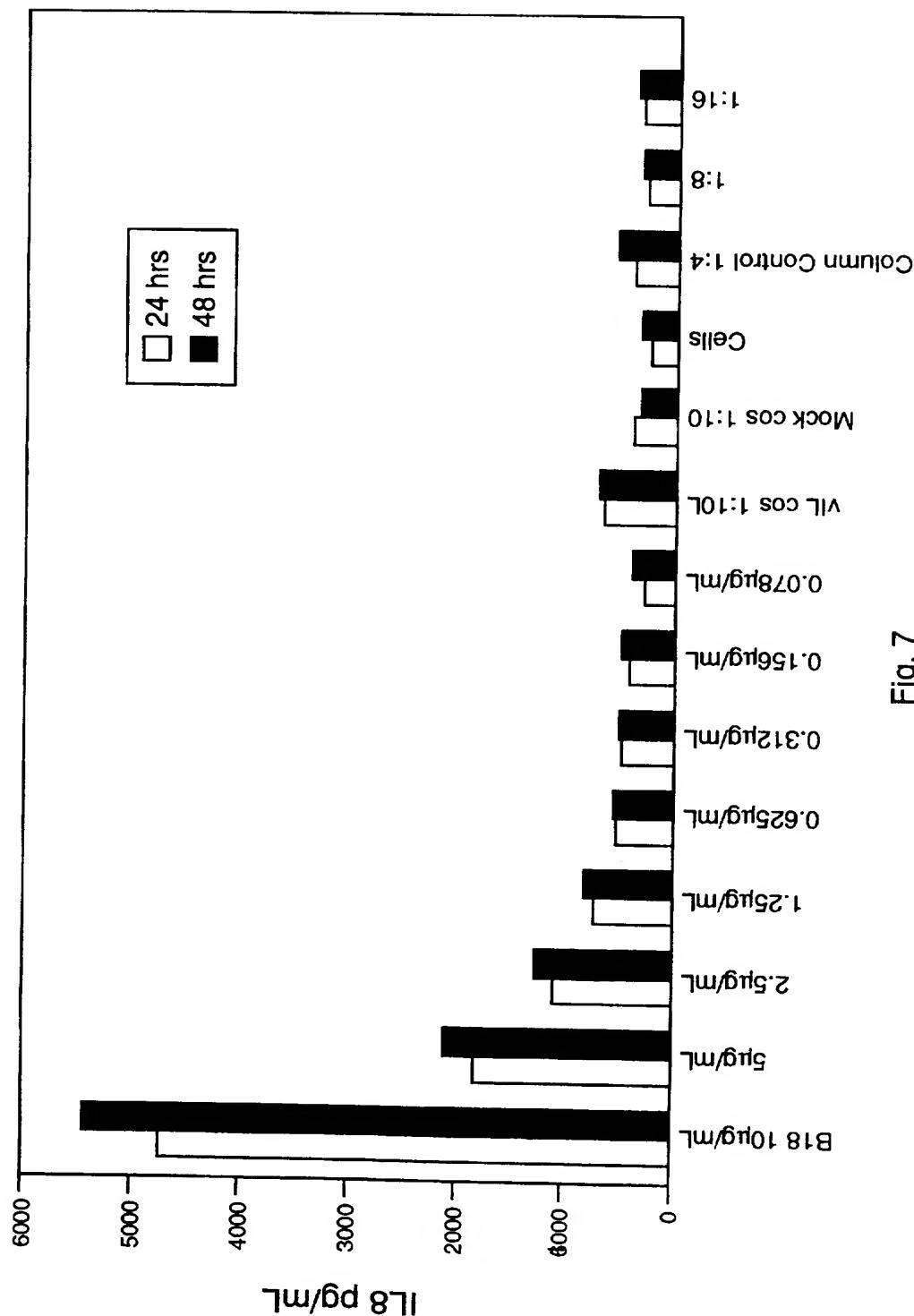


Fig. 7

INTERNATIONAL SEARCH REPORT

I National Application No
PCT/US 96/11889

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/19	C12N15/12	C12N15/24	C07K14/52	C07K14/725
C07K14/54	A61K38/17	A61K38/19	A61K38/20	C12N5/10
C07K16/24	C07K16/28	A61K48/00		

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 18826 A (SCHERING CORP ;INST NAT SANTE RECH MED (FR)) 13 July 1995 cited in the application	1-3,5-7, 12, 15-20, 23-25
A	see page 3, line 21 - page 4, line 11 see page 12, line 1 - page 16, line 6 see page 30, line 1-13 Seq.ID:2 see page 53 Seq.ID:4 see page 55 Seq.ID:5 see page 56 Seq.ID:7/8 see page 58 - page 59 --- -/-	11,13,14



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

1

Date of the actual completion of the international search

21 July 1997

Date of mailing of the international search report

25.07.97

Name and mailing address of the ISA

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Authorized officer

Macchia, G

INTERNATIONAL SEARCH REPORT

National Application No

PCT/US 96/11889

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>THE JOURNAL OF IMMUNOLOGY, vol. 150, no. 12, 15 June 1993, pages 5445-5456, XP002035505 ROUVIER E. ET AL.: "CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a Herpesvirus Saimiri gene" cited in the application see page 5445 - page 5446, line 11 see page 5449, right-hand column, line 26 - page 5450 see page 5451; figure 3 see page 5453, left-hand column, line 10 - right-hand column, line 16 ---</p>	15-20
X	<p>JOURNAL OF VIROLOGY, vol. 66, no. 8, August 1992, pages 5047-5058, XP000615399 ALBRECHT J - C ET AL: "PRIMARY STRUCTURE OF THE HERPESVIRUS SAIMIRI GENOME" Seq.ID:1 from nt.552 to nt.217 (reverse orientation) is 61.4% homologous to X64346 from nt.26931 to nt.27266. see page 5048, right-hand column, line 15-18 ORF13 see page 5049; table 1 ---</p>	1
A	<p>WO 97 07198 A (GENETICS INST) 27 February 1997 see page 8, line 15-31 see page 9, line 26 - page 11, line 7 see page 13, line 16-27 see page 18, line 10 - page 19, line 25 see page 25, line 11-15 see page 27, line 5-17 see page 31, line 27-34 Seq.ID:11-12 see page 49 - page 51 ---</p>	18-20
E	<p>JOURNAL OF IMMUNOLOGY, vol. 155, no. 12, 15 December 1995, pages 5483-5486, XP000602481 YAO Z ET AL: "HUMAN IL-17: A NOVEL CYTOKINE DERIVED FROM T CELLS" see page 5483 see page 5484; figure 1 ---</p>	1-14,21, 22,24-27
P,X	<p>---</p>	1-3,5-7, 23
	-/-	
1		

INTERNATIONAL SEARCH REPORT

National Application No
PCT/US 96/11889

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>THE JOURNAL OF EXPERIMENTAL MEDICINE, vol. 183, no. 6, 1 June 1996, pages 2593-2603, XP002035506</p> <p>FOSSIEZ F. ET AL.: "T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines" see abstract see page 2594; figure 1 ---</p>	1-3,5-7, 15-20, 23,24
P,X	<p>IMMUNITY, vol. 3, no. 6, 1 December 1995, pages 811-821, XP000579309</p> <p>YAO Z ET AL: "HERPESVIRUS SAIMIRI ENCODES A NEW CYTOKINE, IL-17, WHICH BINDS TO A NOVEL CYTOKINE RECEPTOR" see page 811 see page 815, left-hand column, line 50 - page 818, left-hand column ---</p>	15-20, 23,24
E	<p>WO 96 29408 A (IMMUNEX CORP) 26 September 1996 see page 1, line 1 - page 2, line 33 Seq.ID:8 see page 36 ---</p>	18-20
P,X	<p>GENE, vol. 168, no. 2, 12 February 1996, pages 223-225, XP002035631</p> <p>YAO ET AL.: "Complete nucleotide sequence of the mouse CTLA8 gene" see page 225; figure 2 -----</p>	1,2

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/11889

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 14-20, 23, 24

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 14-20, 2, 24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2.

Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3.

Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 96/ 11889

FURTHER INFORMATION CONTINUED FROM PCT/USA/210

- 1) claims 1-14, 21, 22, 25-27 all totally; claim 24 partially.

Isolated polynucleotide comprising Seq.ID:1, homologue sequences and derivates. Vectors and transformed host cells. Process for producing human recombinant CTLA-8 protein. Isolated human CTLA-8 protein as in Seq.ID:2 and fragments. Pharmaceutical compositions and uses in therapy. Antibodies.

- 2) claims 15-17 all totally; claim 24 partially.

Therapeutic uses of the protein as in Seq.ID:4 or fragments.

- 3) claims 18-20 all totally; claim 24 partially.

Therapeutic uses of the protein as in Seq.ID:6 or fragments.

- 4) claims 23 all totally; claim 24 partially.

Therapeutic uses of IL-17 or active fragments.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/11889

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9518826 A	13-07-95	AU 1520895 A EP 0733069 A JP 9501572 T	01-08-95 25-09-96 18-02-97
WO 9707198 A	27-02-97	AU 6712396 A AU 6768596 A WO 9704097 A	18-02-97 12-03-97 06-02-97
WO 9629408 A	26-09-96	AU 5526396 A	08-10-96



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : E01F 9/04, 9/08	A1	(11) International Publication Number: WO 99/04097 (43) International Publication Date: 28 January 1999 (28.01.99)
(21) International Application Number: PCT/US97/23362		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 18 December 1997 (18.12.97)		
(30) Priority Data: 08/895,128 16 July 1997 (16.07.97) US		
(71) Applicant: MINNESOTA MINING AND MANUFACTURING COMPANY [US/US]; 3M Center, P.O. Box 33427, Saint Paul, MN 55133-3427 (US).		
(72) Inventors: JACOBS, Gregory, F.; P.O. Box 33427, Saint Paul, MN 55133-3427 (US). STUMP, Larry, K.; P.O. Box 33427, Saint Paul, MN 55133-3427 (US).		
(74) Agents: CLEVELAND, David, R. et al.; Minnesota Mining and Manufacturing Company, Office of Intellectual Property Counsel, P.O. Box 33427, Saint Paul, MN 55133-3427 (US).		
(54) Title: DIRECTION-INDICATING PAVEMENT MARKING HAVING RAISED PROTUBERANCES AND METHOD OF MAKING		
(57) Abstract		
Retroreflective article (10), e.g., in the form of a pavement marking, having raised, nonintegral protuberances (16) that exhibit good dry retroreflectivity and recover retroreflectivity quickly after exposure to water. The protuberances may comprise a thermoplastic polymer body and have at least two different portions. Partially embedded in the protuberances are different sets of optical elements (14) which are in optical association with a light scattering agent. The protuberances are disposed on top of a conformable base sheet (12).		

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DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

DIRECTION-INDICATING PAVEMENT MARKING**5 HAVING RAISED PROTUBERANCES AND METHOD OF MAKING**TECHNICAL FIELD

The present invention pertains to a direction-indicating retroreflective pavement marking that uses raised protuberances and, partially embedded in the 10 protuberances, a plurality of optical elements having different properties, for example, color or refractive index, to allow the marker to display a different image from a different direction.

BACKGROUND

15 Pavement markings, e.g., paints, tapes, and individually mounted articles, are commonly used to guide and direct motorists traveling along a roadway. During the daytime, the markings are usually sufficiently visible to guide motorists. At nighttime, however, when the primary source of illumination is the vehicle headlights, the marking may not be sufficiently bright to guide the motorist unless it 20 retroreflects light. Retroreflective pavement markings have the ability to return substantial quantities of incident light in the direction from which the light originated. For this reason, retroreflective pavement markings are commonplace on roadways.

Many retroreflective pavement markings, such as lines on highways, are 25 made by dropping optical elements, such as glass beads, onto the line while it is still tacky. Others are made by securing optical elements to a base sheet that contains pigments and fillers. Securement is typically achieved either by embedding the elements into the base sheet or by securing the elements to the base sheet with a binder. The pigments and fillers typically are dispersed throughout the base sheet 30 for several reasons, such as reducing cost, improving durability, and providing conformability. Pigments also enhance pavement marking visibility and can play a role in the retroreflective mechanism.

Incident light retroreflects from pavement markers in the following manner. First, the incident light passes through the optical elements (e.g., microspheres) to strike the pigments in the base sheet or in the bonding material of the marker. The pigments then scatter the incident light back into the microspheres, and the 5 microspheres redirect a portion of the scattered light towards the light source. For effective retroreflection, especially under wet conditions, the microspheres preferably are elevated above the surface of the pavement so that they will not be submerged in water during a rainy period.

An example of a pavement marker where the microspheres are elevated is 10 disclosed in U.S. Patent No. 4,988,555 to Hedblom (referred to as Hedblom '555). This pavement marker contains a pattern of protrusions that have vertical surfaces where microspheres are embedded so as to be elevated above the pavement surface. The microspheres are elevated and are oriented vertical to the incident light, to provide more efficient retroreflection. Because of their elevated position, the 15 microspheres often are not completely submerged in water. The protrusions also allow the water to drain more efficiently from the marker so that retroreflective performance can recover more quickly after rainfall has ceased.

While patterned pavement markers have become very useful articles, their manufacturing process is somewhat complex. For example, as disclosed in U.S. 20 Patent No. 4,988,541 (referred to as Hedblom '541), the integral protrusions are created by embossing a sheet of polymeric material using an embossing roll that has a predetermined pattern of recesses. As the polymeric material fills the recesses in the embossing roll, protrusions having set pattern, dimensions, and spacing are formed. After the embossing process, binder materials are carefully placed on the 25 protrusions in a manner that keeps the binder from flowing into the valleys between the protrusions. Microspheres and/or skid resistant particles are then secured to the binder material. Not only is this process somewhat complicated, but changing the protrusion pattern or shape, size, or spacing requires changing the embossing roll, which typically requires labor and extended amounts of time. With each different 30 protrusion pattern, there must be a corresponding embossing roll.

Hedblom '555 also disclosed a pavement marking that can retroreflect light in two different colors by using different color bead bond layers. The bead bond layers coat the protrusions' vertical sides and support the microspheres. The pigments in the bead bond contribute to the retroreflected light's color. For example, a first bead bond layer facing a first direction can retroreflect white light by using TiO₂ pigment while a second bead bond layer facing a second direction can retroreflect yellow light through the use of lead chromate pigment. The reference also discloses that other color/pigment combinations may be used to provide alternative signal information to drivers.

U.S. Patent No. 4,040,760 (Wyckoff) discloses another example of an enclosed lens direction-indicating pavement marker. The pavement marker has optical elements that are embedded in a polymeric binder layer. This pavement marker has a saw-tooth cross section with each wedge having a relatively long surface inclining upwardly at a small acute angle and a relatively short surface inclining downwardly substantially normal to the upwardly inclining surface. The downwardly inclining surface is disclosed as being reflective, integrally covered, and has a predetermined color. The reflective surface is made by embedding the optical elements with an associated reflective surface in a transparent binder layer. Both the optical elements and the binder may be colored. The upwardly inclining surface has a different color than the downwardly inclining surface and has optically diffuse reflecting properties, such as, for example white paper or flat paint. The reference also discloses that the upwardly inclining surface may be retroreflective. As an example, the downwardly inclining surface retroreflects red light while the upwardly inclining surface scatters white light in all directions. Although this pavement marker may be useful to relay information to a driver, its configuration with enclosed, downwardly inclining surfaces may be relatively difficult to fabricate.

Japanese Patent Kokoku (B2) No. HEI 5[1993]-33661 (Shinmi et al.) discloses a sheet for road signs having optional convex molded shapes on the sheet's surface. Anchored on the convex molded shapes are reflective materials. The sheet comprises thermoplastic polymers and additives, such as fillers, pigments, plasticizers, and reflective materials. The convex molded shapes are made by

forcing molten sheet material into a molding roller. The convex shapes are an integral part of the sheet. The molding roller determines features such as size, shape, and spacing of the convex shapes so that changes to those features cannot be made readily without changing the configuration of the molding roller, a situation similar to Hedblom '541.

International Patent Application No. WO97/18947 discloses a pavement marker that comprises a base sheet, a discontinuous polymeric layer adhered on the base sheet, and a plurality of particles, such as microspheres and skid resistant particles, partially embedded in the polymeric layer. The polymeric layer is a thermoset polymer comprising a blocked isocyanate crosslinker and is applied to the base sheet as a pattern, e.g., a repeating pattern of hexagons, by a continuous process such as screen printing. Although the pavement marker is very useful and although the manufacturing process is generally streamlined, the pattern of the polymeric layer is predetermined by the screen printing method and cannot be changed readily without equipment changes.

An alternate method to elevate the optical elements above the pavement surface is to use retroreflective elements or aggregates having a core material that is coated with a multitude of microspheres. Examples of such elements are disclosed in EP Patent No. 565,756 A2; U.S. Patent Nos. 3,043,196; 3,171827; 3,175,935; 3,274,888; 3,418,896, 3,556,637; 4,983,458; and International Patent Application No. WO95/32337. Although these retroreflective elements are extremely useful, some are not easily manufactured.

SUMMARY OF THE INVENTION

In view of the foregoing, a need still exists for a retroreflective article that can provide good retroreflectivity under wet conditions and that can be direction-indicating while at the same time be manufactured through a streamlined process for making such articles. The present invention provides such a retroreflective article.

The inventive article has a profile of raised protuberances. Optical elements having different properties (for example, color, diameter, refractive index, and composition) are embedded in different portions of the protuberances or in an

optional binder layer disposed on the protuberances. If a binder layer is used, it may have a different color in different portions of the protuberance. Because the protuberances elevate the optical elements from the pavement surface, water drains away from the retroreflective portions of the inventive article more efficiently to 5 allow for a quick recovery of retroreflectivity after a respite of rainfall.

In brief summary, the inventive retroreflective article may comprise or consist essentially of: (a) a base sheet having first and second major surfaces; (b) a plurality of protuberances disposed on the first major surface of the base sheet; (c) at least two sets of optical elements, the first set being partially embedded in a first portion of the protuberances and the second set being partially embedded in a second portion of the protuberances, the second set having different properties than the first set; and (d) at least one light scattering agent that is in optical association with the optical elements such that incident light passing through the optical elements strikes the light scattering agent and is redirected towards its source. 10

The method of the invention may comprise or consist essentially of the steps: (a) providing a base sheet having protuberances and a light scattering agent; (b) partially embedding a first set of optical elements into a first portion of the protuberances such that the optical elements are in optical association with the light scattering agent; and (c) partially embedding a second set of optical elements into a second portion of the protuberances such that the optical elements are in optical 20 association with the light scattering agent, and wherein the second set has different properties from the first set.

Pavement markings of the invention differ from known markings in that they are capable of being easily manufactured and provide an article with protuberances 25 having different sets of optical elements embedded therein. Optical elements of different properties (for example, color, diameter, refractive index, and composition) can be readily deposited on different portions of the protuberances by using, for example, a substantially circular or curved web path for the base sheet and by taking advantage of the protuberance configuration. Unlike the prior art, 30 direction-indicating pavement markings of the invention incorporate an optical system that can be made by using different colored optical elements rather than

different colored binder layers. Because optical elements are more easily selectively secured to the protuberances, the invention provides benefits over known directional protuberance-bearing markers.

In accordance with the invention, articles of the present invention are useful
5 as direction-indicating pavement markers to relay signal information to the motorists, such as warning the motorist that he or she is traveling in the wrong direction.

BRIEF DESCRIPTION OF THE DRAWINGS

10 The invention will be further explained with reference to the drawings, wherein:

Figure 1 is a cross-sectional view showing different portions of a protuberance 16 in accordance with the invention;

15 Figure 2 is a cross-sectional view of retroreflective article 10 in accordance with the invention;

Figure 3 is a cross-sectional view of another embodiment of retroreflective article 20 in accordance with the invention;

Figure 4 is a plan view of the embodiment shown in Figure 3;

20 Figure 5 is a cross-sectional view of another embodiment of retroreflective article 80 in accordance with the invention; and

Figure 6 is a schematic view of a method of making retroreflective article 60 in accordance with the invention.

These figures are idealized, are not to scale, and are intended to be merely illustrative and non-limiting.

25

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Articles of the present invention rely on an optical system comprising sets of optical elements, which can be in the form of microspheres, embedded in different portions of the protuberances. The protuberances are formed on the first major 30 surface of the base sheet. On the second major surface of the base sheet, there are

optional reinforcing layers and adhesive layers to facilitate applying the inventive article to a surface, such as a roadway.

Figure 1 shows an illustrative protuberance 16 of the invention having at least two portions, a first portion 16a and a second portion 16b. Each portion of the protuberance supports different sets of optical elements and can optionally support different color binder layers. The protuberance can support a variety of different sets of optical elements or different colored binder layers as desired.

Figure 2 shows an illustrative embodiment of the invention where a retroreflective article 10 contains a base sheet 12 that has optical element sets 14a and 14b partially embedded in different portions of the protuberances 16 that contain a light scattering agent (not shown).

Figure 3 shows another illustrative embodiment of the invention where a retroreflective article 20 contains a multilayer base sheet 22 having thermoplastic layer 24 containing a light scattering agent (not shown) disposed on conformance layer 26. Protuberances 16 contain a light scattering agent (not shown) and are adhered to thermoplastic layer 24. Optical element sets 14a and 14b are partially embedded in different portions of protuberance 16. Optical elements embedded in thermoplastic layer 24 can be either parts of sets 14a or 14b or both.

Figure 4 shows a plan view of the embodiment in Figure 3 where protuberances 16 of different sizes are scattered randomly on thermoplastic layer 24 of base sheet 22 (not shown). Optical elements, generally shown as 14, are embedded in protuberances 16 and on thermoplastic layer 24 of base sheet 22 (not shown).

Figure 5 shows another illustrative embodiment of the invention where a retroreflective article 80 contains multilayer base sheet 22 having thermoplastic layer 24 disposed on conformance layer 26. Protuberances 16 are coated with binder layer 27, which contains a light scattering agent (not shown). Optical element sets 14a and 14b are partially embedded in binder layer 27 and on thermoplastic layer 24.

The protuberances elevate the optical elements above the substrate surface so that the optical elements are not submerged completely by water, and after a

rainfall, the water can more readily drain away from the protuberances so that they can recover retroreflective performance rapidly. Typically, the protuberance is a raised polymeric core or body. In a preferred embodiment, thermoplastic protuberances are used. As used in this document, the term "thermoplastic protuberance" means a raised body or core that is thermoplastic – that is, capable of melting and flowing when exposed to a sufficient amount of heat. The protuberances optionally can include a binder layer disposed on such core. The binder layer may be a thermoplastic or thermoset polymer. The protuberances have different portions that support different sets of optical elements and optionally support different colored binder layers.

Unlike the protuberances disclosed in U.S. Patent No. 4,988,555 (Hedblom) and Japanese Patent Kokoku (B2) No. HEI 5[1993]-33661 (Shinmi et al.), protuberances of the invention can be nonintegral, meaning that they do not have to be formed as a monolithic element with the base sheet -- that is, the protuberance and base sheet do not need to be formed as a single component that lacks an interface therebetween.

The protuberances provide a profiled structure having a substantially vertical surface resulting in more efficient retroreflection. As between an article with a vertical profile, i.e., an article with protuberances, and one without a vertical profile, i.e., a substantially flat article, an article with a vertical profile provides generally more efficient retroreflection because the optical elements on the vertical surfaces are able to capture more incident light and reflect it back to its source.

The protuberances also provide an enhanced measure of wearability over similarly constructed flat articles without such protuberances. As used in this document, "wearability" means the ability of an article to withstand repeated impact and abrasion from vehicle tires thereby prolonging the useful life of the inventive article. Enhanced wearability of the inventive article is achieved because vehicle tires make contact with the protuberances first, thereby wearing them down before wearing down the remainder of the inventive article.

Illustrative examples of suitable polymers for use as protuberances include fluoropolymer, polycarbonate, acrylic, polyester, polyurethane, polyvinyl chloride,

polyolefin copolymers, and blends thereof. The polymer chosen for the protuberance may be different from that of the thermoplastic layer of a multilayer base sheet. Care should be taken to select a material for the protuberance that has good adhesion to the base sheet, good adhesion to the optical elements, and is
5 sufficiently durable to withstand repeated traffic impact. By "good adhesion" it is meant that the protuberances retain their adhesion to the base sheet and to the optical elements after repeated impact by vehicle tires.

The protuberances can be of essentially any desired shape in horizontal cross section, i.e., in a plane parallel to the marker base sheet when laid flat on a
10 substrate. The horizontal cross section of the protuberance may be, for example, ellipsoidal, circular, oblong, rectangular, irregular, or regular. In some embodiments where optimum retroreflective brightness from all orientations is desired, e.g., a pavement marking for intersections, the horizontal cross section of the protuberance is preferably substantially circular because brightness from all
15 orientations can be achieved. In general, a protuberance having circular cross section creates a substantially hemispherical protuberance, or some portion thereof.

Typically, the protuberances are about 0.2 to about 6.0 millimeters in height and about 1 to 20 millimeters in diameter to be of sufficient size so as not to be submerged completely by water in a typical rain shower. More preferably, the
20 protuberances are about 1 to about 4 millimeters in height and are about 2 to about 8 millimeters in diameter at the base. The latter sizes are more preferred because they tend to provide a good balance between retroreflectivity and wearability, aid in draining water away from the retroreflective portions, and provide a wear surface to prolong the pavement marker's life. Protuberances that are too large may inhibit
25 the retroreflective article's conformance to the substrate, resulting in a reduced adhesive bond. Pavement markers that employ protuberances that may be used in this invention are disclosed in Assignee's copending U.S. Application Serial No. 08/895,132.

As shown in Figure 4, protuberances 16 may be spaced in a generally
30 random manner. The random spacing can be achieved by freely depositing resin particles on the base sheet 12, 22 (Figures 2, 3, and 5). Base sheet 12, 22 may be

heated so that once the polymeric particles contact the base sheet, they soften to yield protuberances of generally hemispherical shape. A less than optimum protuberance spacing may be used in applications where optimum brightness is not required. This feature of random, yet controllable protuberance placement permits 5 a simplified, less expensive manufacturing process to be used in producing a pavement marking that has elevated optical elements. If desired, the protuberances can be in contact with more or less than 50 percent of the surface area of the base sheet, e.g., about 10 to about 40 percent of such surface area.

If desired, to increase optical element adhesion, a binder solution can be 10 coated and cured on the protuberances to yield a binder layer. The binder layer can be a thermoplastic or thermoset polymer. Preferred binder solutions suitable for use in the invention are disclosed in Hedblom '541, which refers to such solutions as "bead bond material." When a binder layer is used, optical elements will be embedded in it and not directly in the protuberances. In such a case, the binder 15 layer contains about 5 to about 20 volume percent of a light scattering agent and the protuberance does not necessarily need to contain light scattering agents. The binder layer may optionally have different colorants on different portions of the protuberances. For example, in Figure 1, a white pigment could be added to the binder solution and coated to a first portion 16a. Similarly a yellow pigment could 20 be added to the binder solution and coated to a second portion 16b. Hedblom '541 discloses preferred coating methods for applying two different binder layers on to a protuberance.

The protuberances can have first and second layers, where the second layer lies under the first layer, the first layer comprising a polymer and a diffuse reflector 25 pigment, and the second layer comprising a polymer and specular pigment (see U.S. Patent No. 5,417,515 (Hachey et al.)). A dual reflecting layer protuberance could be made, for example, using polymeric particles with an inner body comprising a specular pigment and an outer sheath comprising a diffuse pigment. When a polymeric particle of such composition is exposed to heat to form a protuberance, 30 the first outer layer should be of sufficient thickness so that the portion of the optical elements embedded in the protuberance contacts both the outer diffuse

pigment layer and the inner specular pigment layer. Alternatively, a binder layer having a diffuse reflector pigment can be disposed on a protuberance comprising a polymer and a specular reflector pigment.

A dual layer reflecting protuberance can provide high retroreflectivity levels over a wide range of distances and entrance angles, regardless of the retroreflective article's orientation. The specular layer is best suited for returning light that enters close to normal, while the diffuse layer is best suited for returning light at the larger entrance angles between 65° and 90° from normal with respect to the plane formed by the protuberance in contact with the microspheres. Because the protuberance provides a vertical component, higher efficiency of retroreflectivity at driver geometry may be achieved.

Light scattering agents are in optical association with the optical elements. The term "optical association" means that when a light ray strikes the optical element and is refracted, the light ray is capable of striking the light scattering agent so that it can be reflected back into the optical elements. Typically, the light scattering agent resides in a layer that supports the optical elements – that is, in the protuberance or in the binder layer disposed on the protuberance. When the optical elements are partially embedded in the protuberance, the protuberance preferably comprises a light scattering agent. When the optical elements are partially embedded in a binder layer disposed on the protuberance, the binder layer preferably contains a light scattering agent and it is not necessary to have a light scattering agent in the protuberance.

Light scattering agents suitable for use in the present invention include specular pigments and diffuse pigments. Specular pigment particles are generally thin and plate like. Light striking the pigment particles is reflected at an angle equal but opposite to the angle at which it entered. Suitable examples of specular pigments for use in the invention include pearlescent pigments, mica, and nacreous pigments. Diffuse pigments are generally fine particles that are relatively uniform in size. The pigment particles tend to be oriented in many different directions, so that light hitting the particles is reflected back at a number of angles, including back

along the path of incident light. An example of a preferred diffuse pigment is titanium dioxide.

Illustrative examples of suitable light scattering agents for use in protuberances include pigment particles selected from the group consisting of zinc oxide, zinc sulfide, lithophone, zircon, zirconium oxide, barium sulfate, titanium dioxide, and combinations thereof as disclosed in U.S. Patent No. 5,286,682 (Jacobs et al.). These pigments reflect white light. Retroreflective articles using these pigments have the advantage of being able to reflect distinct night time colors without using potentially toxic metals such as cadmium, chromium, and lead-based pigments, when they are used in combination with colored optical elements.

Other light scattering agents may be used to reflect other colors. An illustrative example is bismuth vanadate, which reflects yellow light and can be used with colorless optical elements to yield a yellow pavement marking. Some organic lakes and organic pigments of controlled particle size may also be used.

Typically, the light scattering agents are present at about 5 to 20 volume percent of the layer supporting the optical elements, i.e., the protuberances or the binder layer, if used. Preferably, the scattering agents are present at about 5 to 15 volume percent and more preferably about 7 to about 13 volume percent. This latter range is preferred because it provides a good balance between the amount of scattering agents needed for reflectivity and flowability of the polymeric material during transformation to protuberances. As described above, the light scattering agents can be present in the binder layer, if used, and in the protuberance. Furthermore, different portions of the protuberances or binder layer can comprise different types of light scattering agent. For example, the first portion of the protuberance can comprise a diffuse pigment while the second portion can comprise a specular pigment. Thus, at least one light scattering agent is in optical association with the optical elements. A light scattering agent might also include a layer of specularly reflective material such as a aluminum or silver metal or dielectric layer disposed in optical association with the optical elements.

The base sheets used in the present invention can have a single or a multilayer construction. Whether single or multilayer in construction, the base sheet is desirably conformable so as to be easily applied to a non-planar substrate.

In a multilayer base sheet construction, there is typically a thermoplastic layer disposed on a conformance layer. The conformance layer can be polymeric. U.S. Patent No. 4,490,432 (Jordan) discloses an illustrative conformance layer that is suitable for use in the present invention. This type of conformance layer comprises a non-crosslinked elastomer (e.g., acrylonitrile-butadiene, neoprene, nitrile rubbers, and polyacrylates), a thermoplastic reinforcing polymer (e.g., polyolefins, vinyl copolymers, polyethers, polyacrylates, styrene-acrylonitrile copolymers, polyesters, polyurethanes, and cellulose derivatives), and a particulate inorganic filler (e.g., magnesium silicate, talc, and mica).

Another polymeric conformance layer suitable for use in the present invention is disclosed in U.S. Patent No. 5,194,113 (Lasch et al.) which includes a ductile thermoplastic polymer and a nonreinforcing mineral particulate. This type of conformance layer comprises from about 50 to about 85 volume percent of thermoplastic polymer and about 15 to about 50 volume percent of the mineral particulate, the particulate having a mean particle size of at least one micrometer. Disclosed illustrative examples of suitable thermoplastic polymers include polyolefin, which may be chosen from the group consisting of polyethylene, ethylene copolymers, polypropylene, ethylene-propylene-diene terpolymers, polybutylene, and mixtures thereof. Disclosed illustrative examples of suitable mineral particulate include, e.g., calcium carbonate, aluminum silicate, talc, alumina trihydrate, silica, wollastonite, mica, feldspar, barytes, calcium silicate, attapulgite, and various hollow beads of synthetic and natural minerals.

Yet another polymeric conformance layer suitable for use in the present invention is disclosed in U.S. Patent No. 5,643,655 (Passarino) which is an essentially chlorine-free conformance layer comprising a calandered, unvulcanized compound based on acrylonitrile butadiene rubber (NRB) and modifying agents to make an elastomer precursor. The modifying agents improve the mechanical and physical properties of natural or synthetic elastomer.

The conformance layer can be metallic. Metallic conformance layers should be of sufficient thickness so as to be ductile and conformable and yet have sufficient strength so as to be processable. Illustrative examples of suitable materials for use as metallic conformance layers include aluminum foil and copper foil. Aluminum 5 foil is preferred because it has good conformance properties and is commercially available at a relatively low cost.

For ease of manufacturing, the thermoplastic layer may be laminated to or extruded directly on the conformance layer to yield a multilayer base sheet. In a multilayer construction, it is desirable to have good adhesion between the 10 thermoplastic layer and the conformance layer. Illustrative examples of suitable materials for use as thermoplastic layer include polyolefin copolymers, polyurethane, polyvinyl chloride, and blends thereof. Preferred polyolefin copolymers are ethylene methacrylic acid (EMAA) and ethylene acrylic acid (EAA) because they have very good adhesion to a variety of materials and are 15 commercially available.

The thermoplastic layer may contain light scattering agents similar to those used in the protuberances and binder layer. Typically, the light scattering agents comprise about 5 to about 20 volume percent of the thermoplastic layer. Preferably, light scattering agents are present at about 5 to about 15 volume 20 percent, and more preferably about 7 to about 13 volume percent of the thermoplastic layer to provide a good balance between the amount of scattering agents needed for reflectivity and flowability of the thermoplastic layer during processing. The advantage of having light scattering agents in the thermoplastic layer is that any optical elements embedded therein will also retroreflect incident 25 light. In Figure 3, optical element sets **14a** and **14b** are embedded in protuberances **16** as well as thermoplastic layer **24**, both of which retroreflect incident light. Typically the thermoplastic layer is less than 0.25 mm thick to provide a balance of properties with the conformance layers so as not to substantially inhibit 30 conformability of the inventive article to the substrate. Preferably, the thermoplastic layer is about 0.05 to 0.2 mm thick to strike a good balance between conformability and base sheet integrity.

The optical elements used in the present invention can be light transmissive microspheres. They act as spherical lenses that refract incident light into the protuberances or binder layer which contain the light scattering agent. The light scattering agents reflect a portion of the incident light to direct it back into the 5 microsphere where the light is again refracted but this time back towards the light source.

Different portions of the protuberances or binder layer, if used, support different sets of optical elements. The optical elements can differ in properties such as color, diameter, refractive index, and composition. For example, in Figure 1, 10 protuberance portion **16a** could support colorless optical elements, while in portion **16b**, optical elements could have a yellow transparent colorant. If the protuberance contains a white pigment, for example, titanium dioxide, then portion **16a** should retroreflect white light while portion **16b** should retroreflect yellow light. In this way, a direction-indicating pavement marking can be fabricated.

15 The microspheres can be glass or non-vitreous ceramic. Non-vitreous ceramic microspheres are typically preferred for greater durability and abrasion resistance. Preferred non-vitreous ceramic microspheres are disclosed in U.S. Patent Nos. 4,564,556 (Lange); 4,758,469 (Lange); 4,772,511 (Wood et al.); and 4,931,414 (Wood). Glass microspheres can provide a desirable balance of lesser 20 durability at lower cost. Typically, the microspheres are about 100 to about 600 micrometers in diameter and have a refractive index of about 1.5 to about 2.2.

As shown in Figure 2, the microspheres may be placed only on the protuberances, if desired. Such selective placement is achieved by using a base sheet that is not receptive to the microspheres. A metal conformance layer, such as 25 aluminum foil, is an illustrative example of such a base sheet. Other examples of conformance layers include crosslinked polymers or thermoplastic polymers that have higher melt temperature than the softening point of the protuberances.

The microspheres also can be deposited on the base sheet and the protuberances as shown in Figure 3, where thermoplastic layer **24** of base sheet **22** 30 is receptive to the microspheres. The microspheres can be deposited on a binder layer as shown in Figure 5.

In pavement marking applications, it is important that motorists distinguish between different colored markers, e.g., between white and yellow markers. If desired, light transmissive colorants may be added to the microspheres to enhance both daytime and nighttime color. For example, a yellow colorant could be added 5 to the microspheres to make a pavement marker that retroreflects yellow light. See, for example, U.S. Patent No. 5,286,682 (Jacobs et al.).

Figure 6 shows a process of making retroreflective article 60 of the invention. Reservoir 70 releases polymeric particles 43 containing a light scattering agent (not shown) on to base sheet 42. Heat source 72 begins to soften polymeric 10 particles 43. As base sheet 42 contacts hot can 48 and takes a substantially circular path, polymeric particles 43 further soften, melt, and deform to yield protuberances 16. On the upward portion of the circular path, a first applicator 46 releases a first set of optical elements 14a and with the aid of gravity, the optical elements 14a are partially embedded in a first portion of the protuberances. Some of the optical 15 elements 14a can partially embed in the base sheet if it is receptive to optical elements. The base sheet continues to traverse hot can 48 until it meets a second applicator 54 on the downward portion of the circular path. Applicator 54 releases a second set of optical elements 14b and with the help of gravity, the optical elements are partially embedded in a second portion of the protuberances. Some of 20 the optical elements 14b can partially embed in the base sheet if it is receptive to optical elements. Other methods of partially embedding optical elements into the protuberances include mechanical or pneumatic means. An illustrative example of a pneumatic means is the use of a jet of substantially inert gas to partially embed the optical elements in the protuberances. An illustrative example of a mechanical 25 means is the use of an impeller to throw the optical elements towards the protuberances. Those skilled in the retroreflective arts will take care to position the applicators 46 and 54, as well as to control other processing variables (such as line speed, amount of heat exposure, et cetera) to get the desired coating of optical elements on the protuberances and on the base sheet. If desired, a third applicator 30 could release a third set of optical elements to be partially embedded in a third portion of the protuberances.

Preferably, different sets of optical elements, such as the first set, the second set, and if used, the third set, are of different colors and can also be of different diameters, refractive index, and composition. For example, optical element set 14a can be colorless glass microspheres having a refractive index of about 1.9 while optical element set 14b can be a transparent red ceramic microspheres having a refractive index of about 2.2, both sets of optical elements being partially embedded in a protuberance comprising a white pigment, such as titanium dioxide. In this way, a direction-indicating pavement marking can be made to retroreflect white light in a first direction and red light in a second, opposite direction. Such a pavement marker should be effective to warn motorists from traveling in the second direction.

A variety of heat sources can be used to soften polymeric particles into protuberances. As shown in Figure 6, hot can 48 supplies the heat to soften polymeric particles 43. A heated oven can also be used to soften the particles. For example, as the base sheet moves through an oven, the polymeric particles previously deposited thereon soften, melt, and deform into protuberances. Also, a base sheet carrying polymeric particles can be made to pass under banks of radiant heaters, such as CalrodTM heaters or infrared lamps, to deform the particles into protuberances.

The final shape of the protuberances can vary depending on, for example, (1) the processing conditions such as the temperature and method of heating, (2) the original shapes of the polymeric particles, (3) the melting characteristic of the polymeric particles, and (4) the surface of the base sheet that comes into contact with the polymeric particles. If there is a substantial amount of heat causing the polymeric particles to soften substantially, the final shape of the protuberance may be quite flat. If there is not as much heat, the final shape of the protuberance may be more hemispherical. When the polymer particles are applied randomly and then subsequently heated, some particles may flow together upon heating. Although the resulting protuberance may not have a substantially hemispherical shape, there is generally still significant reflectivity from this now ellipsoidal protuberance.

The density and spacing of the protuberances can be changed easily by changing the base sheet web speed, changing the size of the polymeric particles, or changing the rate of particle deposition.

In the fabrication process, it is typical to add skid resistant particles, if desired, at the same time or just after the optical elements are deposited on to the base sheet. The optical elements and skid resistant particles are applied to the first major surface, i.e., the top surface of the base sheet by, e.g., sprinkling, scattering, et cetera. Examples of conventional skid-resistant particles include corundum (aluminum oxide) and quartz (sand, silicon dioxide, or micronized quartz).

Preferred skid-resistant particles are disclosed in U.S. Patents No. 4,937,127 (Haenggi et al.); 5,053,253 (Haenggi et al.); 5,094,902 (Haenggi et al.); and 5,124,187 (Haenggi et al.).

Components of the inventive article that lie underneath the retroreflective base sheet are preferably selected to fit the application desired. For example, a scrim adhesive (i.e., a polymeric scrim that has been saturated with an adhesive) imparts additional strength, for example, for strength in removability or for other desired wear characteristics, as well as selected adhesive characteristics to the retroreflective article.

20

EXAMPLE

The following example is provided to illustrate different embodiments and details of the invention. Although the example serves this purpose, the particular ingredients and amounts used as well as other conditions and details are not to be construed in a manner that would unduly limit the scope of this invention.

25

A white pavement marking of the invention could be made as follows. Extrude a white film of about 0.11 mm thickness on to a 0.076 mm thick deadsoft aluminum foil carrier to yield a multilayer base sheet by using white resin pellets of NucrelTM 699, an ethylene methacrylic acid copolymer (EMAA), available from Du Pont Company, Wilmington, Delaware, containing 20 weight percent titanium dioxide.

Bring the multilayer base sheet, at a rate of 1.16 m/min, into contact with a hot can having a diameter of about 0.6 m, with the foil side contacting the hot can at a temperature of about 204 °C, sufficiently hot to bring the white film to a nearly molten condition. Sprinkle pigmented cylindrical particles of EMAA, having a 1
5 mm diameter and a 2 mm height and containing 50 weight percent titanium dioxide, on to the EMAA side of the base sheet. Coat the pigmented particles on to the base sheet after about 5 to 8 cm of wrap on the hot can. As the particle coated base sheet continues to traverse the surface of the hot can, the initial cylindrical shape of the particle softens and takes on a generally hemispherical shape to yield
10 protuberances. This base sheet then travels under a first particle coater from which a first set of colorless glass microspheres having about 1.9 refractive index can be sprinkled on the first portion of the protuberance. As the base sheet continues to traverse the hot can, sprinkle a second set of yellow transparent microspheres on to a second portion of the protuberance. Cool the base sheet under ambient condition
15 after it leaves the surface of the hot can and prior to winding it up.

All references cited herein are wholly incorporated by reference in this document.

What is claimed is:

1. A retroreflective article that comprises:
 - (a) a base sheet having first and second major surfaces;
 - 5 (b) a plurality of protuberances disposed on the first major surface of the base sheet;
 - (c) at least two sets of optical elements, the first set being partially embedded in a first portion of the protuberances and the second set being partially embedded in a second portion of the protuberances, the second set having different properties than the first set; and
 - 10 (d) at least one light scattering agent that is in optical association with the optical elements such that incident light passing through the optical elements strikes the light scattering agent and is redirected towards its source.
- 15 2. The retroreflective element of claim 1, further comprising a third set of optical elements in optical association with the light scattering agent, the third set partially embedded in a third portion of the protuberances and having different properties than the first two sets.
- 20 3. The retroreflective element of claim 2, wherein the sets of optical elements are different in at least one property selected from the group consisting of color, size, refractive index, and composition.
- 25 4. The retroreflective article of claim 1, wherein the base sheet comprises a layer selected from the group consisting of ductile metals, thermoset polymers, and thermoplastic polymers having a melting point higher than the softening point of the protuberances.
- 30 5. The retroreflective article of claim 1, wherein the base sheet comprises a conformance layer.

6. The retroreflective article of claim 5, wherein the base sheet further comprises a thermoplastic layer disposed on the conformance layer.

7. The retroreflective article of claim 6, wherein the thermoplastic layer
5 is of a different composition than the thermoplastic material of the protuberances.

8. The retroreflective article of claim 6, wherein the thermoplastic layer
comprises 5 to 20 volume percent light scattering agent.

10 9. The retroreflective article of claim 1, wherein the protuberances
comprise thermoplastic material selected from the group consisting of
fluoropolymer, polycarbonate, acrylic, polyester, polyurethane, polyvinyl chloride,
polyolefin copolymers, and blends thereof.

15 10. The retroreflective article of claim 1, having protuberances with an
average height of 0.2 mm to 6 mm and 1 to 20 millimeters diameter.

20 11. The retroreflective article of claim 1, wherein the protuberances, or a
binder layer disposed on the protuberances, comprise 5 to 20 percent by volume of
a light scattering agent.

25 12. The retroreflective article of claim 1, wherein the light scattering
agent includes pigment particles selected from the group consisting of zinc oxide,
zinc sulfide, lithophone, zircon, zirconium oxide, barium sulfate, titanium dioxide,
pearlescent pigments, mica, nacreous pigments, and combinations thereof.

13. A method for making a retroreflective article comprising:
(a) providing a base sheet having protuberances and a light scattering
agent;

(b) partially embedding a first set of optical elements into a first portion of the protuberances such that the optical elements are in optical association with the light scattering agent; and

(c) partially embedding a second set of optical elements into a second portion of the protuberances such that the optical elements are in optical association with the light scattering agent, and wherein the second set has different properties from the first set.

14. The method of claim 13, further comprising partially embedding a third set of optical elements into a third portion of the protuberances such that the optical elements are in optical association with the light scattering agent, and wherein the third set has different properties from the first two sets of optical elements.

15. The method of claim 13, wherein the sets of optical elements are different in at least one property selected from the group consisting of color, size, refractive index, and composition.

16. The method of claim 13, wherein the protuberances comprise thermoplastic particles.

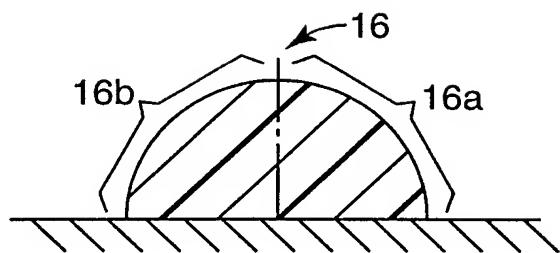
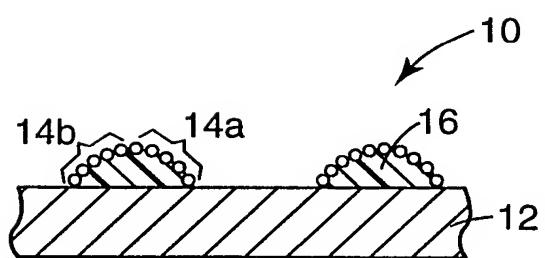
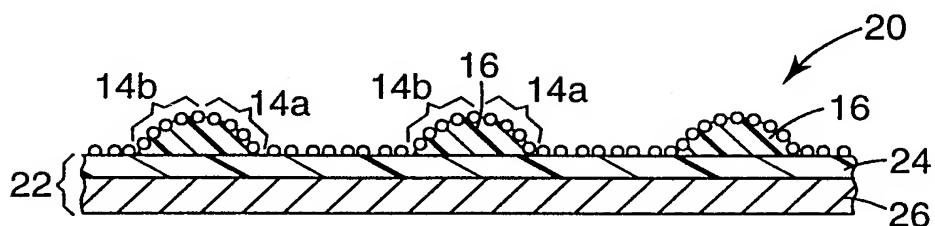
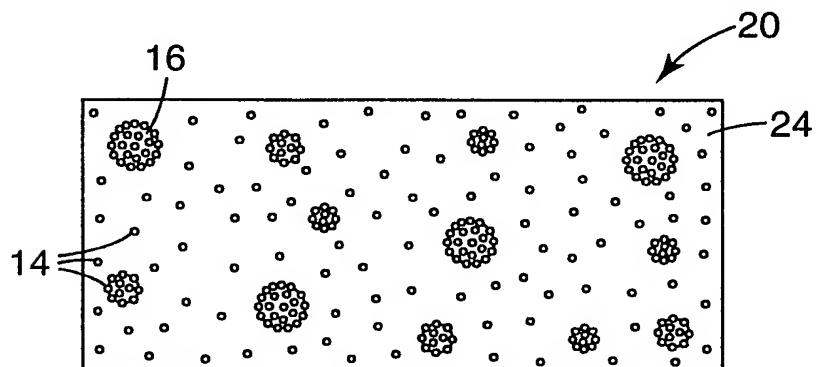
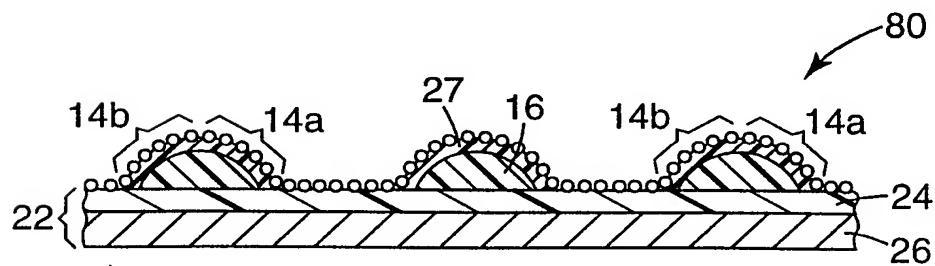
17. The method of claim 13, further comprising heating the protuberances to a softened state so as to be receptive to the optical elements.

25 18. The method of claim 13, further comprising depositing optical elements on the protuberances by use of gravity.

30 19. The method of claim 18, wherein after step (a), the base sheet is allowed to form a substantially circular path where the first set of optical elements is deposited on an upward portion of the circular path and a second set of optical elements is deposited on a downward portion of the circular path.

20. The method of claim 13 further comprising applying a binder layer to the protuberances after step (a) and before steps (b) and (c).

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***Fig. 1******Fig. 2******Fig. 3******Fig. 4******Fig. 5***

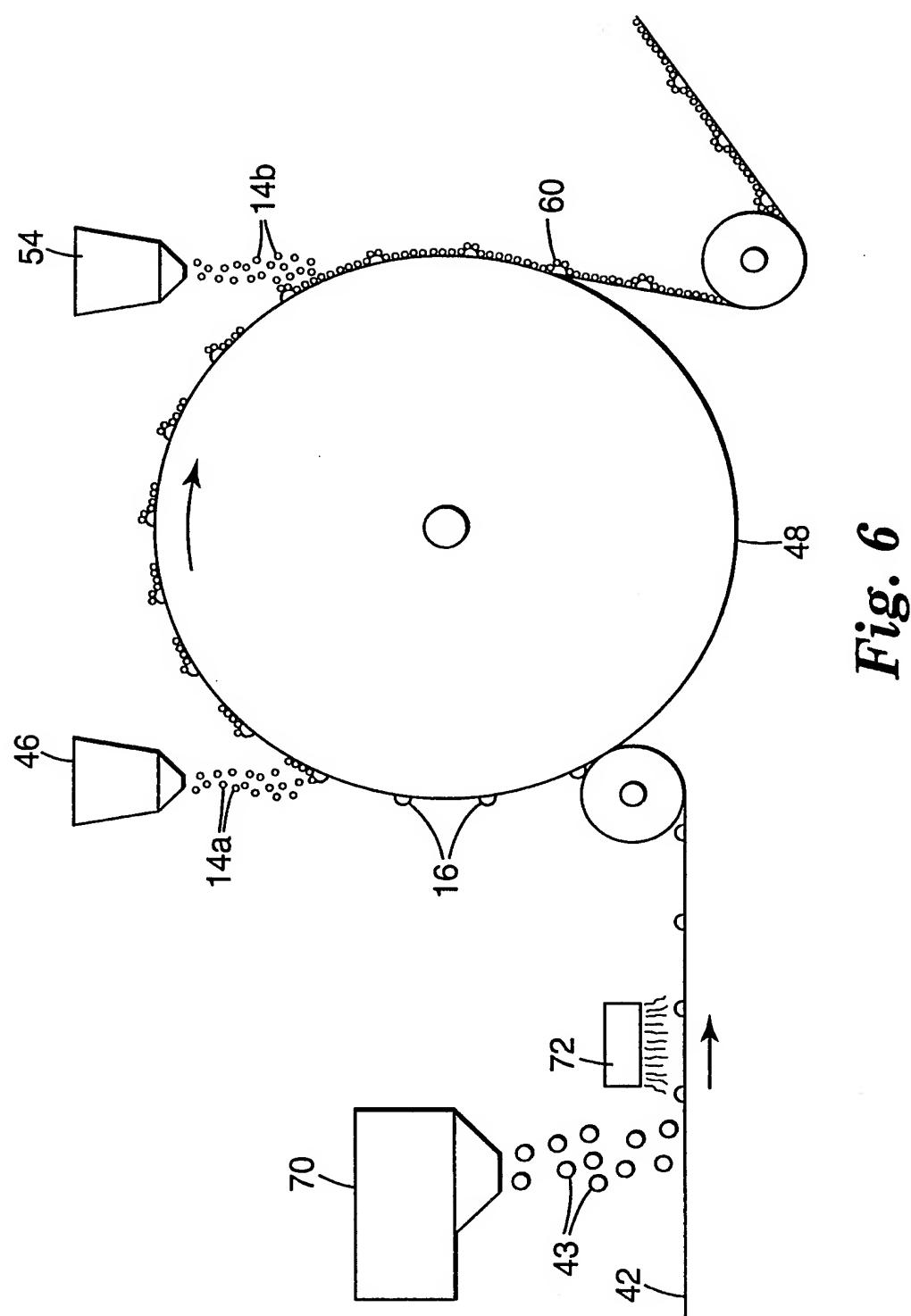


Fig. 6

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/US 97/23362

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 E01F9/04 E01F9/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 E01F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 18947 A (MINNESOTA MINING & MFG) 29 May 1997 cited in the application see the whole document ---	1,4,5,12
A	EP 0 346 021 A (MINNESOTA MINING & MFG) 13 December 1989 see the whole document ---	1,4,10
A	EP 0 683 268 A (MINNESOTA MINING & MFG) 22 November 1995 see the whole document ---	1,9,10
A	US 5 417 515 A (HACHEY KATHLEEN A ET AL) 23 May 1995 cited in the application see the whole document ---	1,11,12
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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

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24 April 1998

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INTERNATIONAL SEARCH REPORT

Inter	nal Application No
PCT/US 97/23362	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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